TRANSLATIONAL CHARACTERIZATION OF ARACHIDONIC ACID METABOLISM IN VASCULAR INFLAMMATION AND CARDIOVASCULAR DISEASE

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ABSTRACT

ROBERT N. SCHUCK: Translational Characterization of Arachidonic Acid Metabolism in Vascular Inflammation and Cardiovascular Disease
(Under the direction of Craig R. Lee, Pharm.D., Ph.D.)

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the United States and novel therapeutic approaches are needed to prevent its development and progression. It is well-established that inflammation is integral to the pathogenesis of coronary artery disease (CAD); therefore, inhibition of the inflammatory response has enormous therapeutic potential to prevent CAD development and progression.

Cytochrome P450 (CYP) enzymes metabolize arachidonic acid to biologically active eicosanoids, and modulation of CYP-mediated arachidonic acid metabolism has emerged as a potential therapeutic target for the treatment of CAD. CYP epoxygenases from the CYP2C and CYP2J subfamilies metabolize arachidonic acid to epoxyeicosatrienoic acids (EETs), which have potent anti-inflammatory effects, and are rapidly hydrolyzed by soluble epoxide hydrolase (sEH) to less biologically active dihydroxyeicosatrienoic acids (DHETs). In contrast, CYP ω-hydroxylase enzymes from the CYP4F and CYP4A subfamilies catalyze the conversion of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) which exhibits potent pro-inflammatory effects. Accumulating evidence indicates that CYP-derived eicosanoids are key regulators of inflammation and endothelial function; however, the functional association between CYP-mediated eicosanoid metabolism, inflammation and endothelial dysfunction, and CAD remains poorly understood.

Therefore, the aim of this dissertation is to characterize the functional contribution of CYP-mediated eicosanoid metabolism to the regulation of systemic and vascular

inflammation within the clinical context of CAD. Using an integrated combination of systems wide and candidate pathway approaches in humans, and pharmacologic and genetic approaches in mice, our findings demonstrate that genes regulating the inflammatory response associate with the presence and severity of CAD. Moreover, we identified a subset of stable CAD patients with enhanced CYP ω-hydroxylase and sEH metabolic function who had advanced endothelial dysfunction and vascular inflammation. In addition, our preclinical studies demonstrated that fatty liver disease-associated inflammation suppressed hepatic CYP epoxygenase activity, and genetic disruption of sEH restored EET levels, and attenuated hepatic and systemic inflammation. Collectively, this dissertation demonstrates that targeted therapeutic strategies that modulate CYP-mediated arachidonic acid metabolism represent a rational anti-inflammatory approach for the treatment of CAD, and thus lays a critical foundation for future studies that directly evaluate the therapeutic effects of modulating CYP-mediated eicosanoid metabolism in CAD patients.

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20-HETE	20-hydroxyeicosatetraenoic acid
5-LO	5-lipoxygenase
Ach	acetylcholine
ACS	acute coronary syndrome
ALOX5	5-lipoxygenase gene
ALOX5AP	5-lipoxygenase activating protein gene
ANOVA	analysis of variance
ApoE	apolipoprotein E gene
ALT	alanine aminotransferase
ATH	atherogenic diet
BH_4	tetrahydrobiopterin
CAD	coronary artery disease
CAM	cellular adhesion molecule
CASP4	caspase-4 gene
CHUK	I-kappa-B kinase-alpha subunit gene
COX	cyclooxygenase
CVD	cardiovascular disease
CYP	cytochrome P450
CYP2C8	cytochrome P450 2C8 gene
Cyp2c29	cytochrome P450 2c29 gene
Cyp2c50	cytochrome P450 2c50 gene
Cyp2c55	cytochrome P450 2c55 gene
CYP2J2	cytochrome P450 2J2 gene
CYP4A11	cytochrome P450 4A11 gene

CYP4F2	cytochrome P450 4F2 gene
DAVID	Database for Annotation, Visualization and Integrated Discovery
DHET	dihydroxyeicosatrienoic acids
DHOME	dihydroxyoctadecenoic
EDTA	ethylenediaminetetraacetic acid
EET	epoxyeicosatrienoic acid
ENA-78	epithelial neutrophil activating protein-78
eNOS	endothelial nitric oxide synthase
EPHX2	soluble epoxide hydrolase gene
EPOME	epoxyoctadecenoic acid
FAS	tumor necrosis factor receptor superfamily member 6 gene
FDR	false discovery rate
FLAP	5-lipoxygenase activating protein
FMD	brachial artery flow-mediated dilation
GEO	gene expression omnibus
GSEA	gene set enrichment analysis
HCV	hepatitis C virus
hs-CRP	high sensitivity C-reactive protein
HIV	human immunodeficiency virus
HLA-E	major histocompatibility complex, class I, E gene
IL-6	interleukin-6
ICAM-1	intracellular adhesion molecule-1
JAK2	Janus kinase 2 gene
KEGG	Kyoto encyclopedia of genes and genomes
LDL	low density lipoprotein
LOX	lipoxygenase

LPS	lipopolysaccharide
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
LTA4H	leukotriene A_4 hydrolase gene
MAPK14	mitogen-activated protein kinase 14 gene
MCP-1	monocyte chemoattractant protein-1
MI	myocardial infarction
MIAME	minimum information about a microarray experiment
Myd88	myeloid differentiation factor 88
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor kappa B
Nfkb1	nuclear factor kappa B p50 subunit gene
NIH	National Institutes of Health
NO	nitric oxide
$ONOO^-$	peroxynitrite
PBMC	peripheral blood mononuclear cell
PLA2G12A	phospholipase A2, group XIIA gene
PRDX3	peroxiredoxin 3 gene
PREDICT	Personalized Risk Evaluation and Diagnosis in the Coronary Tree
PTGIS	prostaglandin-I synthase gene
PTGS1	prostaglandin-endoperoxide synthase 1 gene
PTGS2	prostaglandin-endoperoxide synthase 2 gene
qRT-PCR	quantitative real time-polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species

SAMARA	supporting a multidisciplinary approach to researching atherosclerosis
sEH	soluble epoxide hydrolase
STD	standard diet
t-AUCB	*trans*-4-[4-(3-adamantan-1-ylureido)-cyclohexyloxy]-benzoic acid
TLR	toll-like receptor
Tlr4	toll-like receptor 4 gene
TNFα	tumor necrosis factor alpha
TBXAS1	thromboxane A synthase 1 gene
TXA_2	thromboxane A_2
UNC	University of North Carolina-Chapel Hill
VCAM-1	vascular cellular adhesion molecule-1
WT	wild-type

CHAPTER I

INTRODUCTION

Clinical Problem

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the United States and novel therapeutic approaches are needed to prevent its development and progression. In 2008, over 800,000 individuals died from cardiovascular causes in the United States, which accounts for 1 in every 3 deaths.[1] Coronary artery disease (CAD) is defined as the buildup of atherosclerotic plaque in the vessel wall of the coronary arteries. The progression of CAD results in narrowing of the vessel lumen, plaque instability, and ultimately plaque rupture and an acute coronary syndrome (ACS) clinical event. Over 1.2 million people in the United States will experience a new or recurrent ACS event each year, making it a substantial burden on the healthcare system.[1] In recent decades considerable progress has been made in the development of therapeutics for the treatment of CAD; however, mortality remains high and over 36% of men and 47% of women will die within 5 years following a myocardial infarction (MI).[1] The extensive morbidity and mortality associated with CAD highlights the need for new therapies that target biological pathways integral to the pathogenesis and progression of CAD. However, a more thorough understanding of the mechanisms underlying the progression of CAD is necessary to facilitate the development of new therapeutic strategies that mitigate CAD progression, prevent ACS clinical events and cardiovascular death, and improve public health.

Inflammation is a Key Pathological Mediator of CAD

Atherosclerosis is a chronic disease that begins with the recruitment of monocytes to the coronary artery wall leading to the infiltration of macrophages, development of foam cells and formation of plaque, proliferation of the atherosclerotic lesion, and ultimately plaque rupture and ACS clinical events.[2] The pathogenesis of CAD is complex, involving intricate interactions between the vessel wall and circulating blood cells; however, it is clear that innate immune system-mediated inflammatory responses play a key pathological role in all stages of atherogenesis.[3] Most notably, activation of the transcription factor nuclear factor-kappa B (NF-κB) through toll-like receptors (TLRs) and its downstream signaling is central to the regulation of inflammation and the pathogenesis and progression of atherosclerosis.

Vascular Inflammation

Vascular inflammation is integral to the development and progression of CAD.[4] In the early stages of atherosclerosis, activation of NF-κB results in endothelial activation and increased expression of key pro-inflammatory cytokines (i.e., interleukin-6 [IL-6] and tumor necrosis factor alpha [TNFα]), chemokines (i.e., monocyte chemoattractant protein-1 [MCP-1]), and cellular adhesion molecules (CAMs; i.e., intracellular adhesion molecule [ICAM]-1 and vascular cellular adhesion molecule [VCAM]-1) on the endothelium.[5] Cytokines are pro-inflammatory signaling molecules that drive the inflammatory response,[6] chemokines are chemoattractant cytokines that recruit leukocytes to the vessel wall[7] and CAMs mediate leukocyte adherence to the endothelium.[8] Collectively, these pro-inflammatory mediators facilitate the migration, adherence and infiltration of monocytes into the vessel wall where they differentiate into macrophages and engulf lipoproteins to form foam cells, a key step in the development of the atherosclerotic lesion.[9] Circulating IL-6 promotes the release C-reactive protein (hs-CRP) from the liver, initiating a systemic inflammatory response that propagates the vascular inflammatory response (Figure 1.1).[9] These inflammatory

processes also contribute to plaque instability by breaking down the extracellular matrix; ultimately resulting in plaque rupture, formation of a thrombus, occlusion of the vessel, and an ACS clinical event.[2]

Recent studies using preclinical models have demonstrated that inhibition of vascular inflammation may be a viable therapeutic strategy to prevent the development and progression of atherosclerosis. For example, LDL receptor knockout mice lacking the chemokine MCP-1 (*Ldlr*$^{-/-}$/*MCP-1*$^{-/-}$) develop significantly less atherosclerosis than *Ldlr*$^{-/-}$ mice wild-type (WT) for MCP-1.[10] Similarly, mice deficient in both apolipoprotein E (*ApoE*$^{-/-}$) and the CAMs ICAM-1 (*ApoE*$^{-/-}$/*ICAM-1*$^{-/-}$), E-selectin (*ApoE*$^{-/-}$/*E-selectin*$^{-/-}$), or P-selectin (*ApoE*$^{-/-}$/*P-selectin*$^{-/-}$) have significantly lower atherosclerotic plaque lesion areas than *ApoE*$^{-/-}$ littermates.[11] Collectively, these data indicate that endothelial activation and the subsequent increase in chemokines and CAMs are integral to the development and progression of CAD, and anti-inflammatory therapeutic strategies that attenuate NF-κB mediated vascular inflammation, may attenuate the development and progression of CAD.

In humans, it has become well-established that circulating biomarkers of systemic and vascular inflammation are predictive of CAD risk, as well as prognosis in patients with established CAD. For example, higher circulating levels of CAMs, including VCAM-1, ICAM-1 and E-selectin, are predictive of death from cardiovascular causes[12] Similarly, elevated MCP-1 levels are associated with higher risk of all-cause mortality in patients with established CAD.[13] The role of neutrophils in atherosclerosis has recently become appreciated,[14] leading to the study of neutrophil chemokines including epithelial neutrophil activating protein (ENA)-78[15] as potential mediators of vascular inflammation in patients with CAD. Genetic variation in ENA-78 has been associated with higher plasma ENA-78 levels[16] and higher rates of all-cause mortality in ACS patients.[17] Collectively, these data demonstrate that biomarkers of vascular inflammation are critical in the pathogenesis and progression of CAD and are valuable phenotypic endpoints in pre-clinical and human

studies. Consequently, adjunct therapies that attenuate vascular inflammation have enormous therapeutic potential to prevent the progression of atherosclerosis and improve prognosis in CAD patients.

Endothelial Dysfunction

Endothelial dysfunction is a physiologic manifestation of vascular inflammation secondary to increased oxidative stress and impaired nitric oxide (NO) availability and function. Endothelial activation, and the subsequent macrophage infiltration into the vascular wall during the early stages of atherogenesis, results in increased production of reactive oxygen species (ROS) within the endothelium. Under conditions of oxidative stress $NO\cdot$ and $O_2\cdot^-$ react to produce peroxynitrite ($ONOO^-$) which diminishes levels of tetrahydrobiopterin (BH_4).[18] BH_4 is an essential cofactor for the enzyme endothelial nitric oxide synthase (eNOS), the main source of NO in the vasculature, and insufficient levels result in eNOS producing $O_2\cdot^-$ rather than NO.[18] This process is referred to as eNOS uncoupling and results in further reduction of NO availability, ultimately leading to endothelial dysfunction.[19] Impaired physiologic measures of endothelial function, most notably brachial artery flow-mediated dilation (FMD), are associated with future cardiovascular events in multiple patient populations.[20, 21] Moreover, CAD patients with persistently impaired FMD despite optimized therapy have a higher risk of cardiovascular events.[22] Collectively, these studies illustrate that endothelial dysfunction is a key pathological mediator of CAD, and suggest that adjunct therapies that attenuate vascular inflammation and/or improve FMD may subsequently improve prognosis.

Systemic and Hepatic Inflammation

Vascular inflammation mediated by cytokines, chemokines, and CAMs leads to endothelial dysfunction and is a key pathological mediator of CAD. However, systemic

inflammation can propagate the inflammatory response in the vasculature by increasing circulating levels of pro-inflammatory cytokines leading to more advanced vascular inflammation and endothelial dysfunction (Figure 1.1). Hepatocytes play a key pathological role in the propagation of the inflammatory response by releasing numerous pro-inflammatory mediators into the systemic circulation including cytokines, chemokines and acute-phase reactants.[23] Most notably, circulating IL-6 signals the liver to synthesize hs-CRP, a potent acute-phase reactant that initiates and propagates a potent inflammatory cascade.[9] Elevated hs-CRP is associated with increased risk of CAD development, recurrent MI and death from cardiovascular causes in individuals with established CAD, and is used clinically to assess cardiovascular risk and guide treatment,[4, 24, 25] indicating that the systemic inflammatory response is important in the development and progression of CAD and therapeutic strategies that mitigate the systemic inflammatory response may improve prognosis in CAD patients.

Non-alcoholic Fatty Liver Disease/Non-alcoholic Steatohepatitis

The liver is a key mediator of the initiation and propagation of the systemic inflammatory response and regulation of cholesterol homeostasis. Given the integral role of hepatocytes in the inflammatory response, and the potent pro-inflammatory effects of cholesterol, it has recently become appreciated that patients with chronic liver disease also have enhanced systemic and vascular inflammation.[26] Non-alcoholic fatty liver disease (NAFLD) effects approximately 30% of the population, and ranges from simple steatosis to steatosis combined with inflammation (non-alcoholic steatohepatitis [NASH]), and may progress to cirrhosis and fibrosis of the liver.[27] The severity of NAFLD has been associated with higher CVD risk, and the presence of hepatic inflammation (NASH) confers the highest CVD risk in both male and female subjects.[28] Importantly, the association between NAFLD and CVD risk persists after adjusting for cardiovascular risk factors including age, sex, smoking history,

diabetes duration, HbA1c, LDL cholesterol, liver enzymes, medication use, and the metabolic syndrome, suggesting the presence of a pathological link between NAFLD and CVD rather than simply shared risk factors.[29] Since inflammation plays a key role in the development and progression of both NAFLD and CVD, it is hypothesized that increased oxidative stress and NF-κB activation secondary to the presence of fatty liver disease drives vascular inflammation, endothelial dysfunction and the subsequent development and progression of CAD.[30] This pathological link between NAFLD and CAD is supported by interventional studies in preclinical models and humans demonstrating that direct administration of free fatty acids increased plasma free fatty acid levels (a hallmark of NAFLD), resulted in NF-κB activation and impaired NO production in endothelial cells and elevated circulating levels of ICAM-1 and VCAM-1.[31, 32] This direct functional interaction between fatty liver disease-associated systemic and vascular inflammation and CVD in preclinical models and humans suggests that novel therapeutic strategies that attenuate fatty liver disease-associated inflammatory response have enormous therapeutic potential to subsequently attenuate systemic and vascular inflammation and the development and progression of CAD.

Summary and Significance

Systemic and vascular inflammation, and the subsequent development of endothelial dysfunction, are integral to the pathogenesis and progression of CAD. Consequently, biomarkers of inflammation and endothelial function that are predictive of prognosis are valuable phenotypic endpoints in pre-clinical and human studies, and therapeutic strategies that decrease systemic or vascular inflammation and improve endothelial function have enormous potential to slow disease progression and improve outcomes in CAD patients. However, recent failures in drug development indicate that new therapeutics are unlikely to be effective in broadly defined populations. Therefore, identification and rigorous

characterization of the pathways central to the regulation of NF-κB dependent vascular and hepatic inflammatory responses in preclinical models and humans will be critical to facilitate the development of novel anti-inflammatory therapeutic strategies for the treatment of CAD, and identify putative responders to these therapeutic strategies.

Arachidonic Acid Metabolism

Metabolism of arachidonic acid to biologically active eicosanoids is critical in the regulation of numerous biological processes including inflammation.[33, 34] Arachidonic acid is obtained through dietary sources or synthesized from linoleic acid and stored in cell membrane phospholipids where it is subsequently released by cytosolic phospholipase A_2, the key regulator of free arachidonic acid levels.[35] Free arachidonic acid is metabolized by the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzyme families to bioactive fatty acids known as eicosanoids (Figure 1.2).[36]

Cyclooxygenase Pathway

The most widely studied pathway of arachidonic acid metabolism is the COX pathway, which plays a role in the regulation of inflammation and other biological processes.[37] COX-1 is expressed on platelets where, in conjunction with thromboxane A_2 (TXA_2) synthase, it metabolizes arachidonic acid to TXA_2, a potent inducer of platelet aggregation.[38, 39] Inhibition of COX-1 with low-dose aspirin prevents arachidonic acid induced platelet aggregation, reduces risk of cardiovascular death, MI and stroke, and therefore is standard of care in CAD patients.[40, 41]

COX-2 is expressed in the vasculature and on macrophages and metabolizes arachidonic acid to pro-inflammatory prostaglandin E_2 (PGE_2) and anti-inflammatory prostaglandin I_2 (PGI_2).[37, 42] Inhibition of COX-2 has yielded conflicting effects on cardiovascular inflammatory responses and atherosclerosis in pre-clinical models.[42] For

example, the COX-2 inhibitor celecoxib increased TNFα expression in macrophages treated with lipopolysaccharide (LPS), and increased atherosclerotic lesion area in *ApoE*$^{-/-}$ mice treated with LPS.[43] Conversely, genetic disruption of COX-2 in macrophages attenuated LPS induced expression of MCP-1 and TNFα, and the COX-2 inhibitors rofecoxib and NS-398 reduced early atherosclerotic lesion formation in female *ApoE*$^{-/-}$ mice.[44] These preclinical findings highlight the complex role of COX metabolism and prostaglandin signaling in the regulation of inflammation and CVD development and progression. Importantly, data in humans have clearly shown that selective inhibition of COX-2, significantly increases the risk of thrombosis and CAD clinical events,[45-47] whereas non-selective inhibition of COX-1/2 with non-steroidal anti-inflammatory drugs (NSAIDs) is not associated with clear evidence of protection or harm in CVD patients.[48] Therefore, selective inhibition of COX-2 is relatively contraindicated, and routine use of non-selective NSAIDs is not recommended, in CAD patients secondary to increased risk of cardiovascular events and the associated morbidity and mortality.[49] The complex role of COX-mediated eicosanoid metabolism in CVD, and the established role of low-dose aspirin in the secondary prevention of CAD, highlights the need for better understanding of parallel pathways of arachidonic acid metabolism in CAD.

5-Lipoxygenase Pathway

The LOX pathway metabolizes arachidonic acid to biologically active eicosanoids, including leukotrienes.[34] 5-lipoxygeanse (5-LO) and its activating protein (FLAP) are highly expressed in leukocytes and in the liver, and both enzymes are required to metabolize the conversion of arachidonic acid to leukotriene A_4.[50] Leukotriene A_4 is subsequently converted to LTB_4, a potent leukocyte activator and chemoattractant, or cysteinyl leukotrienes, which mediate bronchoconstriction and have other biological effects.[51] In contrast to COX derived eicosanoids, which have both pro- and anti-inflammatory effects, leukotrienes are clearly

pro-inflammatory in both acute and chronic models of inflammation. Mice with genetic disruption of the gene encoding 5-LO (*Alox5*$^{-/-}$) have attenuated induction of inflammation and reduced organ damage following LPS administration.[52] Similarly, *ApoE*$^{-/-}$/*Alox5*$^{-/-}$ double-knockout mice demonstrate reduced hepatic inflammation compared to *ApoE*$^{-/-}$ control mice[53] and pharmacologic inhibition of FLAP attenuated adipose tissue inflammation in response to a high-fat diet.[54] Despite the clear role of 5-LO pathway in the pathogenesis of inflammation, inhibition of 5-LO mediated LTB_4 biosynthesis has yielded conflicting results in preclinical models of atherosclerosis.[55] Initial reports demonstrated that mice heterozygous for the 5-LO null mutation on an *Ldlr*$^{-/-}$ background (*Alox5*$^{-/+}$/*Ldlr*$^{-/-}$) had reduced atherosclerotic lesion area;[56] however, larger subsequent studies of *Alox5*$^{-/-}$ mice on *Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$ backgrounds failed to replicate this finding.[57, 58] Similarly, pharmacologic inhibition of the 5-LO pathway has produced inconsistent effects on atherosclerotic lesion development.[58, 59] Consistent with preclinical data, initial findings that genetic polymorphisms in the 5-LO pathway were associated with subclinical CAD[60] have not been shown to be associated with clinical events,[61] and clinical trials evaluating 5-LO pathway inhibition have produced only modest effects on biomarkers of inflammation in CAD patients.[62, 63] Collectively, these data support a modest role for the 5-LO pathway in the pathogenesis of CAD, and further demonstrate the need for better understanding of parallel pathways of arachidonic acid metabolism in CAD

Cytochrome P450 pathway

CYP enzymes are predominately expressed in the liver where they catalyze the oxidative biotransformation of most exogenous drugs and other xenobiotics.[64] Regulation of CYP expression and metabolic activity are impacted by genetic polymorphisms, hormones, the presence of certain disease states, and pro-inflammatory cytokines.[64] In addition to their important role in xenobiotic metabolism in the liver, certain CYP isoforms are expressed in

extrahepatic tissues and metabolize endogenous substrates including arachidonic acid.[65] This "3rd pathway" of arachidonic acid metabolism catalyzes the conversion of arachidonic acid to biologically active eicosanoids in multiple tissues and cell types.

CYP Epoxygenase pathway

CYP epoxygenase enzymes from the CYP2C and CYP2J subfamilies are expressed in endothelial cells, cardiomyocytes and hepatocytes where they metabolize arachidonic acid to the biologically active 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid (EET) regioisomers; however, EETs are quickly hydrolyzed by soluble epoxide hydrolase (sEH) to their corresponding dihydroxyeicosatrienoic acids (DHETs), which have much less biological activity.[66] CYP epoxygenase derived EETs elicit vasodilatory,[67, 68] fibrinolytic,[69] pro-angiogenic,[70] anti-apoptotic,[71] and cardioprotective effects[72, 73] by a variety of mechanisms.

Most notably, EETs exert potent anti-inflammatory effects in the cardiovascular system.[74] A seminal paper published by Node, et. al. in 1999 was the first to demonstrate that pretreatment of human umbilical vein endothelial cells with 11,12-EET attenuated the TNFα induced increase in expression of ICAM-1, VCAM-1, and E-selectin.[75] Additional *in vitro* studies have demonstrated that direct administration of EETs attenuates endothelial activation via decreased activation of the key pro-inflammatory transcription factor NF-κB by inhibiting IκB kinase (IKK) activity and the subsequent phosphorylation and ubiquitination of inhibitor κB-α (IκB-α) and translocation of NF-κB to the nucleus.[74] In addition, several studies indicate that potentiation of the CYP epoxygenase pathway may represent an effective anti-inflammatory therapeutic strategy *in vivo*. Following LPS administration, mice treated with the sEH inhibitor AUDA-BE had lower plasma levels of IL-6 and TNFα as well as monocyte chemoattractant protein-5 compared to vehicle treated control mice.[76] Similarly, we observed that endothelial over-expression of human *CYP2J2* or *CYP2C8* or global disruption of murine *Ephx2*, attenuates the LPS-mediated induction of pro-inflammatory

cytokine (IL-6 and IL-1β), chemokine (MCP-1 and ENA-78) and CAM (E-selectin) expression, and neutrophil infiltration into lung tissue, in mice via decreased activation of NF-κB.[77] Inhibition of sEH has also exhibited protective effects in chronic models of CVD. *ApoE*$^{-/-}$ mice infused with angiotensin II had lower rates of abdominal aortic aneurysm formation and reduced atherosclerotic lesion area when treated with a sEH inhibitor (AR9276).[78] Notably, these effects were associated with reduced aortic expression of cytokines and CAMs, and circulating levels of inflammatory biomarkers, suggesting they were mediated by the anti-inflammatory properties of sEH inhibition. The vasodilatory and anti-inflammatory properties of EETs have led to the development of pharmacologic inhibitors of sEH for the treatment of human CVD.[79] Importantly, the protective effects of EETs in preclinical models of atherosclerosis and inflammation suggest that pharmacologic inhibition of sEH may have clinical utility as an adjunct anti-inflammatory therapeutic strategy for the treatment of CAD.

Genetic polymorphisms in the CYP epoxygenase pathway have been associated with CAD risk in humans.[80] For example, a polymorphism in *EPHX2* (K55R) is associated with higher epoxide hydrolase activity *in vivo* (lower EETs) and higher risk of CAD.[81] In addition, we observed that the *EPHX2* K55R polymorphism is associated with endothelial dysfunction in Caucasians.[82] Similarly, a *CYP2J2* promoter polymorphism (*CYP2J2 -50G>T*) is associated with lower *CYP2J2* promoter activity *in vitro*, lower circulating DHETs and higher risk of CAD in humans;[83] and, the *CYP2C8* K399R variant allele has lower CYP epoxygenase activity *in vitro*[84] and is associated with higher risk of ACS clinical events.[85] However, the strength of associations between genetic variants in CYP-mediated eicosanoid metabolism and risk of CAD development is not consistent across all studies,[80] suggesting that the relationship between CYP-mediated arachidonic acid metabolism and CAD is complex, and likely most profound in certain subsets of the population. Moreover, we recently reported that clinical factors including obesity, age, diabetes, and cigarette smoking

are associated with CYP epoxygenase and sEH metabolic function, indicating that non-genetic factors may also impact the relationship between CYP-mediated eicosanoid metabolism and CAD risk. Despite these initial observations in humans, the contribution of inter-individual variation in CYP epoxygenase and sEH metabolic function to inflammation and endothelial dysfunction in CAD patients remains poorly understood and requires rigorous evaluation.

CYP ω-hydroxylase Pathway

In contrast to the vascular-protective effects of the CYP epoxygenase pathway, human CYP ω-hydroxylase enzymes from the CYP4F and CYP4A subfamilies catalyze the conversion of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE)[86] which exhibits potent vasoconstrictive and pro-inflammatory effects in preclinical models.[86] For example, the addition of 20-HETE to endothelial cells results in the activation of NF-κB, increased production of pro-inflammatory CAMs, and increased superoxide and decreased NO production secondary to eNOS uncoupling.[87, 88] In rat renal interlobar arteries 20-HETE impairs acetylcholine induced vasorelaxation and the CYP ω-hydroxylase inhibitor HET0016 improves vasorelaxation in 5α-dihydrotestosterone treated animals via increased NO bioavailability.[89] In addition, *in vivo* studies have demonstrated that endothelial over-expression of CYP4A2 in Sprague-Dawley rats results in increased blood pressure and impaired vasorelaxation in response to acetylcholine via increased 20-HETE biosynthesis.[90] Furthermore, the putative 20-HETE receptor antagonist 20-6,15-HEDE attenuates the 5α-dihydrotestosterone induced decrease in acetylcholine-induced vasorelaxation and increase in NF-κB activation in renal interlobar arteries.[91]

In humans, genetic epidemiology studies have demonstrated that the CYP ω-hydroxylase pathway is associated with CVD risk. For instance, presence of the *CYP4F2* V433M minor allele is associated with higher renal excretion of 20-HETE and higher

incidence of hypertension and ischemic stroke, indicating that 20-HETE may be an important mediator of vascular tone in humans.[92, 93] Similarly, higher urinary excretion of 20-HETE has also been associated with lower FMD in a subjects without known CAD.[94] Despite these initial observations in humans, the functional relationship between inter-individual variation in CYP ω-hydroxylase mediated eicosanoid metabolism and inflammation and endothelial dysfunction has not been rigorously evaluated, and requires further study.

Summary and Significance

Emerging evidence has demonstrated that CYP-derived eicosanoids are key regulators of inflammation and endothelial function in preclinical models. Pharmacologic agents that increase EETs or decrease 20-HETE elicit potent protective effects in the cardiovascular system, and represent potentially promising adjunct anti-inflammatory therapeutic strategies for the treatment of CAD. However, the functional role of CYP-mediated eicosanoid metabolism in fatty liver disease-associated inflammatory responses, which are increasingly recognized as key contributors to the pathogenesis and progression of CAD, remains unknown. Therefore, preclinical studies are necessary to define the contribution of CYP-mediated eicosanoid metabolism to fatty liver disease-associated systemic and vascular inflammation.

Furthermore, although genetic epidemiology studies suggest that CYP-mediated arachidonic acid metabolism may be important in the pathogenesis of CAD in humans, the functional contribution of CYP-derived eicosanoids to the regulation of biological processes underlying the pathogenesis and progression of CAD in humans remains largely unknown. Therefore, studies evaluating the association between inter-individual variation in CYP-mediated eicosanoid metabolism and biological processes integral to the pathogenesis and progression of CAD, including inflammation and endothelial dysfunction, are necessary to

provide further insight into the functional relevance of CYP-mediated eicosanoid metabolism in humans and its contribution to the pathogenesis and progression of CAD.

Perspective

The significant morbidity and mortality associated with CAD highlights the need to better understand the mechanisms underlying the pathogenesis and progression of atherosclerosis in order to facilitate the development of novel therapeutic strategies that mitigate these key pathological mechanisms, and thus offer the potential to improve outcomes. Accumulating evidence clearly indicates that systemic and vascular inflammation drive the pathogenesis and progression of CAD. Therefore, discovery and rigorous characterization of the key mechanisms underlying this fundamental pathological process, in both preclinical models and humans, offers enormous potential to develop rational therapeutic strategies that are likely to improve clinical outcomes. Based on emerging evidence indicating CYP-derived eicosanoids are key regulators of inflammation and endothelial function, we hypothesize that modulation of CYP-mediated eicosanoid metabolism offers enormous therapeutic potential in CAD patients. However, the functional association between CYP-mediated eicosanoid metabolism, inflammation and endothelial dysfunction, and CAD remains poorly understood, and requires rigorous investigation in preclinical models and humans.

Thus, the overall aim of this dissertation is to characterize the functional contribution of CYP-mediated eicosanoid metabolism to the regulation of systemic and vascular inflammation within the clinical context of CAD. We seek to accomplish this aim using an integrated combination of systems biology (Specific Aim I) and candidate pathway (Specific Aim II) approaches in humans with CAD and pharmacologic and genetic approaches in mice (Specific Aim III) following induction of a systemic and vascular inflammatory response relevant to human CAD.

Completion of this dissertation will serve to improve our understanding of the functional role of CYP-mediated eicosanoid metabolism in the pathogenesis and progression of CAD, provide further mechanistic insight into the impact of therapeutic strategies that modulate CYP-mediated arachidonic acid metabolism on systemic and vascular inflammation, and thus lay a critical foundation for future studies that directly evaluate the therapeutic effects of modulating CYP-mediated eicosanoid metabolism in CAD patients. Furthermore, these studies offer enormous potential to identify a population of putative responders to anti-inflammatory therapeutic strategies that modulate CYP-mediated eicosanoid metabolism.

Specific Aims

1) Utilize a global systems biology approach to identify the key biological processes that underlie the development and progression of obstructive CAD in humans.

Overall Hypothesis: Global gene expression analyses in peripheral blood mononuclear cells (PBMCs) will demonstrate that pathways involved in innate immune-mediated inflammatory responses are the most important biological processes in the development and progression of obstructive CAD in humans

Specific Hypotheses:

a) Expression of genes that mediate the innate immune response in PBMCs will be dysregulated in humans with obstructive CAD.

b) Expression of genes that metabolize arachidonic acid to biologically active eicosanoids in PBMCs will be dysregulated in humans with obstructive CAD.

2) Utilize a candidate pathway approach to characterize the functional relationship between inter-individual variation in CYP-mediated eicosanoid metabolism and endothelial dysfunction, vascular inflammation, and systemic inflammation in stable obstructive CAD patients.

Overall Hypothesis: CYP-mediated eicosanoid metabolism is important in the regulation of endothelial dysfunction and vascular and systemic inflammation in stable, obstructive CAD patients.

Specific Hypotheses:

a) Impaired CYP epoxygenase and enhanced sEH metabolic function (i.e. lower EETs) is associated with endothelial dysfunction, advanced vascular inflammation, and advanced systemic inflammation in patients with stable, obstructive CAD.

b) Enhanced CYP ω-hydroxylase metabolic function (i.e. higher 20-HETE) is associated with endothelial dysfunction, advanced vascular inflammation, and advanced systemic inflammation in patients with stable, obstructive CAD.

3) Utilize a preclinical approach to define the contribution of CYP-mediated eicosanoid metabolism to the regulation of non-alcoholic fatty liver disease-associated systemic and vascular inflammation in mice.

Overall Hypothesis: CYP-mediated eicosanoid metabolism is an integral mediator of fatty liver disease-associated systemic and vascular inflammation *in vivo*.

Specific Hypotheses:

a) Induction of fatty liver disease will significantly suppress CYP epoxygenase-mediated EET biosynthesis *in vivo* via activation of the innate immune system and suppression of hepatic CYP epoxygenase expression.

b) Inhibition of sEH mediated EET hydrolysis will restore EET levels and attenuate fatty liver disease-associated systemic and vascular inflammation *in vivo*.

Figures

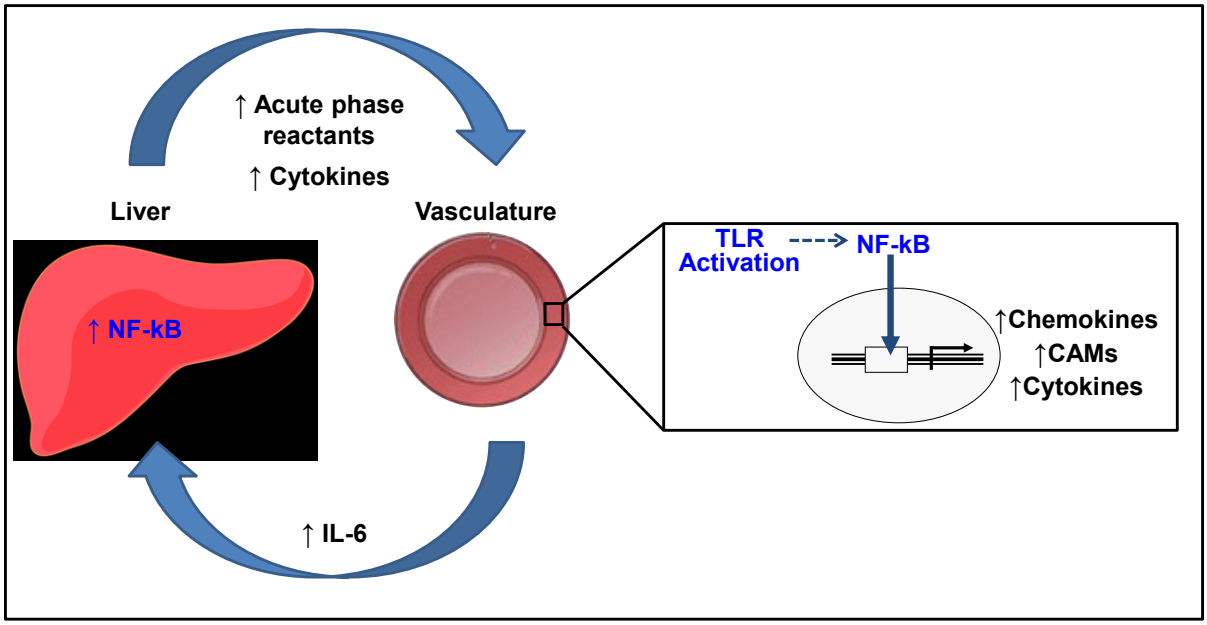

Figure 1.1. Interaction between hepatic, systemic, and vascular inflammation.

The innate immune response is initially activated in the liver secondary to cholesterol and lipid accumulation or in the vasculature by macrophages following TLR activation. Upon activation of the innate immune response, a positive feedback loop is created in which IL-6 that is formed in the vasculature is released into systemic circulation, and signals the liver to synthesize acute-phase reactants (e.g. C-reactive protein) and additional and cytokines (e.g. IL-6), chemokines (e.g. MCP-1), and CAMs (e.g. ICAM-1). Release of these pro-inflammatory mediators from the liver propagates the inflammatory response in the vasculature through further activation of NF-κB and drives endothelial activation, increased biosynthesis of cytokines, chemokines, and CAMs, and the subsequent development and progression of atherosclerosis.

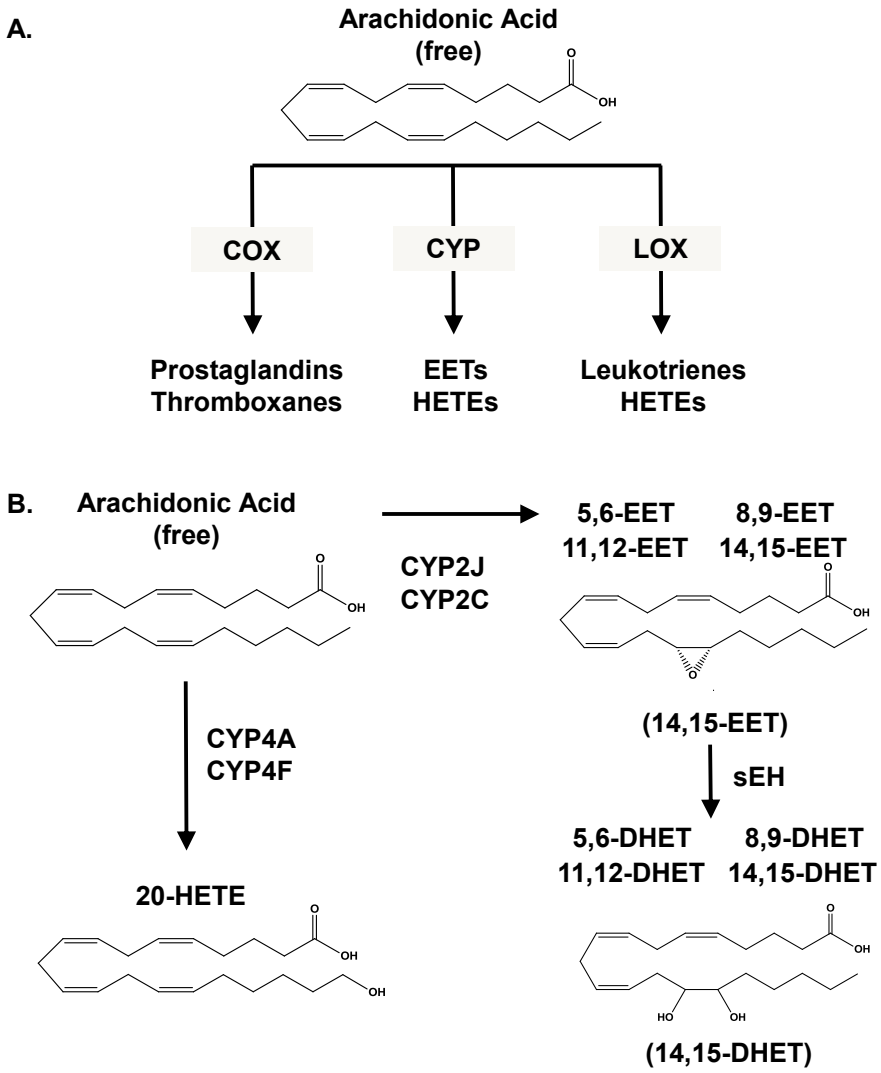

Figure 1.2. Overview of arachidonic acid metabolism.

(A) After release from the cell membrane by cytosolic phospholipase A_2, free arachidonic acid is metabolized by enzymes from the COX, LOX, and CYP pathways to biologically active eicosanoids.

(B) Arachidonic acid is metabolized by CYP ω-hydroxylases from the CYP4A and CYP4F subfamilies to 20-HETE or by CYP epoxygenases from the CYP2C and CYP2J subfamilies to one of four EET regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET). The EETs (epoxides) are subsequently hydrolyzed by sEH to their corresponding DHET (diol) metabolites, which generally have less biological activity. Structures for the 14,15-EET regioisomer, the preferred EET substrate for sEH, and the 14,15-DHET metabolite are provided since the 14,15-EET:DHET (epoxide:diol) ratio is an established biomarker of sEH metabolic function.

CHAPTER II

INFLAMMATORY BIOMARKERS AND GLOBAL GENE EXPRESSION IN OBSTRUCTIVE CORONARY ARTERY DISEASE

Introduction

It is well-established that age is associated with cardiovascular disease (CVD) risk. Epidemiology studies have demonstrated that the lifetime risk for CVD remains high in older individuals and increases dramatically in the presence of multiple risk factors.[95] In addition, older individuals who experience an acute coronary syndrome (ACS) suffer poorer prognosis compared to the general population. For example, in patients age 65 or older, the five year mortality rate following a myocardial infarction (MI) is 46-58% and chances of death, recurrent MI or fatal coronary heart disease, and stroke are all higher compared to younger individuals.[1] However, older patients are frequently under-represented in clinical trials and the pathophysiology underlying the development and progression of atherosclerotic coronary artery disease (CAD) has not been rigorously evaluated in this population. Increasing age is associated with pathophysiological changes in the vasculature including increased carotid intima-media thickness, arterial stiffening, endothelial dysfunction, and elevated systolic blood pressure.[96] While it is well-established that systemic and vascular inflammation are important in the development and progression of CAD in the general population,[4, 9] studies in older patient populations have not consistently demonstrated that elevated biomarkers of inflammation are associated with poorer outcomes.[97, 98] These findings suggest that the pathophysiology underlying CAD in older individuals may be distinct from that in younger individuals. Therefore, identification of the

key biological processes underlying the development and progression of CAD in this high-risk patient population may facilitate the development of novel therapeutic strategies to improve prognosis.

Systems biology studies have identified global gene expression signatures in peripheral blood mononuclear cells (PBMCs) that are predictive of the presence and extent of coronary artery atherosclerosis,[99, 100] suggesting that evaluation of gene expression in PBMCs may provide insight into the key biological processes underlying the development and progression of CAD. In addition, gene expression changes that occur in peripheral blood have been reported to mirror gene expression changes in atherosclerotic arteries.[100] Therefore, evaluation of global gene expression changes in PBMCs, and application of functional annotation tools to identify the key biological processes dysregulated in the presence of CAD, represents a non-biased approach to elucidate the key mechanisms underlying the development and progression of CAD.

Although elevated circulating biomarkers of inflammation including C-reactive protein (hs-CRP), monocyte chemoattractant protein-1 (MCP-1) and cellular adhesion molecules (CAMs) have been repeatedly associated with the development and progression of CAD in the general population,[9] and poor prognosis in patients with established CAD,[12, 13, 24] the relationship between inflammation and CAD risk is less clear in older adults. Furthermore, the key biological processes underlying the pathogenesis and progression of CAD in older individuals, including the contribution of circulating inflammatory biomarkers, remains unknown. Therefore, in order to improve our understanding of the pathogenesis and progression of CAD in older adults, we utilized a 1) candidate biomarker approach to determine if the presence and severity of obstructive CAD is associated with elevated circulating biomarkers of inflammation and 2) systems biology approach to identify gene expression changes and dysregulated biological pathways in PBMCs that are associated with the presence and severity of obstructive CAD in older adults.

Methods

Study Population

The Supporting A Multidisciplinary Approach to Researching Atherosclerosis (SAMARA) study enrolled consecutive individuals who were at least 65 years of age and undergoing diagnostic cardiac catheterization as part of their routine care. Between March 2007 and February 2009 we enrolled 143 individuals from the University of North Carolina (UNC) Cardiac Catheterization Laboratory. Individuals with HIV or HCV infection, lymphoma, leukemia, anemia, history of rheumatoid arthritis or lupus, history of solid organ transplantation and individuals undergoing current cancer treatment or chronic immunosuppressive therapy were not eligible to participate. Participants were interviewed to obtain pertinent medical information including previous medical history, current medication use, family medical history, and smoking status. This study was approved by the UNC Institutional Review Board and conducted according to institutional guidelines; all participants provided written informed consent.

CAD Severity

Subjects underwent diagnostic left-heart catheterization as part of their routine medical care. Following the procedure, CAD severity was quantified in each subject by coronary angiography by calculating a CAD score as follows: 0 (<10% stenosis in all major coronary arteries); 1 (10-70% stenosis in at least one vessel); 2 (>70% stenosis in one vessel); 3 (>70% stenosis in two vessels); and, 4 (>70% stenosis in 3 vessels or >70% stenosis in the left-main coronary artery).

Obstructive CAD was defined as >70% stenosis in ≥1 major epicardial coronary artery (CAD score 2-4). Subjects with ≤70% occlusion in any vessel (CAD score 0-1) were considered free of obstructive CAD and thus were utilized as the comparator group.

Sample Collection and Processing

All catheterizations took place in the morning following an overnight fast; at the start of the procedure 30 mL of blood was drawn from the femoral artery into Na-EDTA Vacutainer tubes (Becton, Dickinson and Co., Cockeysville, MD). Following blood collection RNA and DNA were isolated from PBMCs as described.[101] RNA was purified using the RNeasy mini-kit (Qiagen, Valencia, CA). RNA quantity, purity and integrity were assessed by spectrophotometry and microcapillary electrophoresis on an Agilent BioAnalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). Only RNA with an A260/280 ratio of ≥ 1.8, and an electrophoretic profile consistent with only minimal degradation was considered acceptable for use. An additional 8 mL of blood collected in tubes containing EDTA and proteinase inhibitor was processed to plasma, aliquoted, and stored at -80ºC pending analysis.

Gene Expression Profiling

RNA was co-hybridized to Agilent G4112A Whole Human Genome 44K oligonucleotide arrays in the presence of Cyanine-3 labeled Universal Human Reference RNA (UHRR, Stratagene, LaJolla, CA) for array normalization, as described.[101] Slides were hybridized and washed, then scanned on an Axon 4000b microarray scanner; data were processed using Agilent software. Only probes flagged “detected” in at least 85% of all samples were included, resulting in 18,411 probes in the final dataset. In addition, only samples with at least 90% of probes flagged detected were included in the analyses. Missing data were imputed using the k-nearest neighbor algorithm (k=10). MIAME-compliant datasets were deposited with the Gene Expression Omnibus of the National Center for Biotechnology Information (GEO Series accession number GSE12959).

Microarray expression data was corrected to account for scan-date batch effects using the remove batch-effect feature in Partek Genomics Suite v6.6. Unsupervised analyses determined the primary signal contributing to gene expression was gender. In addition, diabetes was found to be a potential confounder to CAD severity. Therefore we conditioned the dataset using an analysis of variance (ANOVA) linear model to account for gender and diabetes in order to identify potential probes that correlated with CAD severity.

Quantification of Circulating Biomarkers of Inflammation

Plasma concentrations of CAMs (E-selectin, P-selectin, intracellular adhesion molecule [ICAM]-1, and vascular cellular adhesion molecule [VCAM]-1) and neutrophil (epithelial neutrophil-activating protein-[ENA]-78) and monocyte (MCP-1) chemokines were quantified using the Human Adhesion Molecule and Human Cytokine Fluorokine® Multi-Analyte Profiling Kits (R&D Systems, Minneapolis, MN), respectively. hs-CRP was quantified using the Human C-Reactive Protein Fluorokine® MAP kit for use in Cardiac Panel B. All analytes were quantified with fluorescence detection on the Bio-Plex 200 System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Comparison of Functional Pathways to Independent CAD Cohorts

In order to compare the gene expression signatures identified in our analysis to those previously identified in CAD patients, we accessed the published list of 655 genes that were associated with CAD in the case:control analyses from both the CATHGEN[102] and Personalized Risk Evaluation and Diagnosis in the Coronary Tree (PREDICT)[103] cohorts.[104] The CATHGEN cohort identified 87 CAD cases (defined as ≥75% stenosis in one major coronary vessel or ≥50% stenosis in two major coronary vessels) who were 63 ± 10 years of age and 108 control subjects (defined as <25% stenosis in all major coronary vessels) who were 55 ± 11 years of age. The PREDICT cohort enrolled only non-diabetic individuals and

identified 99 CAD cases (defined as ≥50% stenosis in at least one major coronary artery) who were 62 ±11 years of age and 99 age and gender matched controls (defined as <50% stenosis in all major vessels) who were 55 ± 12 years of age. The association between gene expression and case:control status in these studies was assessed using logistic regression for the CATHGEN cohort and conditional logistic regression for the PREDICT cohort.

Statistical Analysis

Circulating Biomarkers of Inflammation

Demographic and clinical characteristics were compared in subjects with and without obstructive CAD using regression for continuous variables and chi-squared analysis for categorical variables. All variables that were not normally distributed were log-transformed prior to analysis. Since hs-CRP was above the limit of detection in 53 (37%) of the samples analyzed, a value of 15.2 mg/L (1.5-fold higher than the highest standard) was imputed for each sample above the upper limit of detection.

Spearman's rank correlation was used to determine the correlation among circulating biomarkers of inflammation. Multiple CAMs were significantly correlated (data not shown); therefore, in order to minimize redundancy in our analysis, a consolidated 'CAM score' phenotype was calculated in each individual, as described,[105] by summing the z-scores of E-selectin, P-selectin, ICAM-1, and VCAM-1. None of the remaining biomarkers of inflammation (hs-CRP, MCP-1, ENA-78, CAM score) were significantly correlated.

The association between plasma levels of the selected inflammatory biomarkers and CAD score was evaluated using an unadjusted regression model and a model that adjusted for potential demographic (age, race, gender) and clinical (obesity, diabetes mellitus, hyperlipidemia, statin use) confounders that associated with biomarkers of inflammation or obstructive CAD status in the SAMARA cohort. Similarly, plasma levels of these inflammatory biomarkers were compared in subjects with and without obstructive CAD using

both the unadjusted and adjusted regression models. A secondary analysis compared the proportion of subjects with high (>median) or low (≤median) inflammatory biomarker levels in individuals with and without obstructive CAD using a Chi-square analysis. For hs-CRP, an additional analysis was conducted comparing the proportion of individuals with and without obstructive CAD who had hs-CRP levels >3 mg/L, the clinically relevant threshold that confers higher risk of CAD.[106] All statistical analyses were conducted using SAS Version 9.3 (SAS, Cary, NC).

Global Gene Expression Profiling

Spearman rank correlation was utilized to generate a list of genes that correlated with the CAD score (0-4) with a predefined cutoff of $r_s \geq 0.25$ or $r_s \leq -0.25$ using Partek Genomics Suite (Partek Incorporated, St. Louis, MO). Pathway enrichment was determined using the National Institutes of Health (NIH) Database for Annotation, Visualization and Integrated Discovery (DAVID) Version 6.7, which uses a modified Fisher's Exact Test to calculate an "Enrichment Score" for each functional annotation cluster.[107, 108] Biological Process Terms and Molecular Function Terms were included as Gene Ontologies, and the Classification Stringency was set to high; all other default parameters were unchanged. The gene list obtained from our correlation analysis was uploaded to DAVID and compared to the human genome reference to generate a list of functional pathways that were over-represented with increasing CAD severity. P-values were calculated by computing $10^{-\text{Enrichment Score}}$, with $p<0.05$ (Enrichment Score >1.3) considered statistically significant. A similar analysis was conducted using the list of 655 genes that were differentially expressed in subjects with and without CAD in both the CATHGEN and PREDICT studies.[104] This allowed us to compare the functional pathways identified in our analysis of older individuals in the SAMARA cohort to the functional pathways from previously published cohorts.

Results

Study Population

The study population characteristics stratified by obstructive CAD status are shown in Table 2.1. A total of 93 subjects (65%) had obstructive CAD while 50 (35%) did not have obstructive CAD. In both groups the majority of subjects were Caucasian, and had multiple comorbidities including hyperlipidemia and hypertension. A significantly higher proportion of those diagnosed with obstructive CAD were male, had a previous diagnosis of hyperlipidemia and myocardial infarction, and were taking medications for the treatment of cardiovascular disease including statins, aspirin, and clopidogrel (Table 2.1).

Candidate Biomarkers of Inflammation (plasma)

Plasma concentrations of hs-CRP and MCP-1, and the CAM score, were not significantly associated with CAD score (Table 2.2). Moreover, when analyzed by the presence or absence of obstructive CAD, no significant differences in hs-CRP, MCP-1, and CAM score were observed in subjects with obstructive CAD compared to subjects without obstructive CAD in either the unadjusted or adjusted models (Table 2.3). In contrast, ENA-78 levels were inversely associated with CAD score (Table 2.2), and were significantly lower in subjects with obstructive CAD in both the unadjusted and adjusted models (Table 2.3, Figure 2.1). Similarly, the proportion of subjects with high (above the median) levels of hs-CRP, MCP-1, and CAMs was not significantly different in subjects with and without obstructive CAD, while subjects with obstructive CAD were significantly more likely to have low ENA-78 levels (Table 2.4). In addition, individuals with obstructive CAD were not significantly more likely to have hs-CRP levels greater than 3 mg/L (Table 2.4).

Since we did not observe a significant positive association between any of the candidate biomarkers of inflammation and either CAD score or the presence of obstructive CAD, we sought to develop a more comprehensive phenotypic index of inflammation by integrating

hs-CRP, MCP-1, and ICAM-1 into a single 'inflammation score' phenotype (Appendix I). Clear differences in this 'inflammation score' were observed between a cohort of patients with angiographically-confirmed CAD and healthy volunteers at low risk for CAD, and thus enabled us to more completely assess the association between presence and severity of obstructive CAD and inflammation in the SAMARA cohort. Similar to the analysis of individual inflammatory biomarkers, however, the 'inflammation score' was not significantly associated with CAD score or the presence of obstructive CAD in the SAMARA cohort (Appendix I).

Global Gene Expression (PBMCs)

Our global gene expression analysis identified 708 probes that significantly correlated with CAD severity (CAD score 0-4) with $r_s \geq 0.25$ or $r_s \leq -0.25$ (Figure 2.2). The absolute correlation coefficient (r_s) of ≥0.25 corresponded to a p-value ≤0.0026 in our analysis; only 48 probes would pass this threshold based on chance, indicating only a small proportion of our findings are likely due to chance. Of the 708 probes, 364 were positively correlated with CAD score while 344 were inversely correlated, indicating that increasing severity of CAD is associated with higher expression of certain genes in PBMCs, but lower expression of others. The 708 probes identified in our analysis corresponded to 512 unique annotated genes (Gene List 1). Functional analysis using DAVID identified 28 annotation clusters that were significantly enriched ($p<0.05$) in our list of 512 unique annotated genes. The 10 functional annotation clusters with the highest enrichment scores are presented in Table 2.5. A description of each functional annotation cluster "representative annotation terms" and the number of genes in each functional annotation cluster that were included in the gene list are provided. The fold enrichment (number of genes in the functional annotation cluster relative to the number that would be expected by chance) of each functional annotation cluster in the gene list and the p-value are provided.

The top annotation cluster represented genes involving immune system development. Fifteen genes from this pathway were positively correlated with the CAD score while 5 were inversely correlated with the CAD score (Figure 2.3), indicating that genes involved in immune system development were expressed at higher levels in subjects with more severe CAD. Other highly enriched annotation clusters included antigen and protein processing, actin related genes, and processes involved in the regulation of transcription (Table 2.5); the majority of genes from these pathways were positively correlated with the CAD score (Figure 2.3). Of the 512 unique annotated genes that were correlated with the CAD score, 89 were a component of at least one functional annotation cluster (Gene List 2). A heat map displaying the association between CAD score and each of the 89 genes included in the functional annotation clusters is displayed in Figure 2.4.

Comparison of Functional Pathways to Independent CAD Cohorts

We compared our list of 512 unique annotated genes to the 655 genes that significantly associated with CAD in the case:control analyses from both the CATHGEN and PREDICT studies. There were 45 genes identified in our study that were also identified in both the CATHGEN and PREDICT cohort, which is 2.7-fold greater than the number of overlapping genes expected by chance ($p<0.001$), indicating that we were able to identify gene expression changes in our cohort of older individuals that are similar to previously validated gene expression changes in younger populations (Gene List 3). Our functional analysis of the gene list from the CATHGEN and PREDICT studies identified 23 annotation clusters that were significantly enriched ($p<0.05$). The 10 functional annotation clusters from the CATHGEN/PREDICT analysis with the highest enrichment scores are presented in Table 2.6. Although no identical annotation clusters were represented in top 10 processes from both the CATHGEN/PREDICT analysis and the analysis of the SAMARA cohort, clusters representing pathways involved in regulation of the immune response were represented in

both analyses. Three annotation clusters representing genes involved in the regulation of apoptosis were also identified in the top 10 functional annotation clusters from the CATHGEN/PREDICT analysis.

Discussion

Inflammation is integral to the development and progression of CAD in the general population; however, the role of inflammation in the pathophysiology underlying the development and progression of CAD in older patient populations has remained largely unexplored. Our analysis of candidate circulating inflammatory biomarkers, which are critical mediators of systemic and vascular inflammation and capture the inflammatory response at multiple levels, did not demonstrate a significant association between higher levels of inflammation and the presence and severity of obstructive CAD in older individuals. However, our global gene expression analysis in PBMCs revealed that genes regulating the immune response are expressed at higher levels in subjects with more severe CAD. These findings suggest that although elevated circulating candidate biomarkers of inflammation were not significantly associated with the presence and severity of obstructive CAD in older individuals, inflammation signaling is a key pathological factor underlying the pathogenesis and progression of CAD in this patient population. Moreover, global gene expression changes in PBMCs may be more reflective of the underlying pathology of CAD in older individuals than circulating protein levels, suggesting that circulating biomarkers of inflammation may have less clinical utility for assessment of cardiovascular risk and prognosis in this patient population.

In order to characterize the role of systemic and vascular inflammation in the pathophysiology of CAD in older individuals, we first evaluated the association between the presence of obstructive CAD and well-established circulating mediators of inflammation (hs-CRP, CAMs, MCP-1, and ENA-78) that have been associated with higher CAD incidence in

the general population,[109-111] and higher mortality in patients with established CAD.[12, 13, 17, 24] Our analysis demonstrated that hs-CRP (an acute phase reactant reflective of systemic inflammation), MCP-1 (a chemokine synthesized in monocytes and endothelial cells that drives monocyte recruitment to the vascular wall), and CAMs (inflammatory mediators expressed on endothelial cells that mediate leukocyte and platelet adhesion) were not associated with the presence or severity of obstructive CAD in a population of older adults presenting for coronary angiography. Although circulating biomarkers of inflammation are frequently elevated in CAD patients compared to healthy individuals and are associated with CAD incidence,[4] the association between inflammatory biomarkers and CAD in older individuals is less clear. For example, an analysis of over 4,500 individuals over 65 years of age demonstrated only a modest association between hs-CRP levels and CVD events (hazard ratio of 1.13, 95% confidence interval 1.05-1.21 for every standard deviation increase).[112] Moreover, a study of more than 1,200 individuals over 65 years of age showed that changes in hs-CRP levels over three years, but not baseline levels, were associated with all-cause mortality.[97] Collectively, these data indicate that circulating biomarkers of inflammation have insufficient power to differentiate between older adults with and without obstructive CAD, and to predict prognosis in older patients with established CAD, suggesting a limited role in risk assessment of older individuals.

Surprisingly, the neutrophil chemokine ENA-78 was inversely associated with CAD score, and ENA-78 levels were significantly lower in subjects with obstructive CAD compared to subjects without obstructive CAD. Although genetic predisposition to higher ENA-78 levels has been associated with poorer survival in ACS patients, the association between plasma ENA-78 levels and the presence of obstructive CAD in humans has not been evaluated to date. Our findings demonstrate that elevated ENA-78 levels in older individuals are not associated with the presence and severity of obstructive CAD. Given the association between genetic predisposition to higher ENA-78 levels and higher mortality in

ACS patients,[17] and preclinical studies demonstrating that neutrophil recruitment to the vascular wall promotes atherosclerotic plaque formation in mice,[113] it is possible that ENA-78 is suppressed in obstructive CAD patients via a compensatory response. However, less is known about the role of neutrophil chemokines in the development and progression of atherosclerotic CAD in humans, and future studies evaluating the contribution of neutrophil recruitment to the vascular wall to the pathogenesis and progression of CAD in older adults are warranted.

Our candidate biomarker analysis has limitations that must be acknowledged. Although the circulating biomarkers of inflammation that we quantified have been associated with CAD risk in younger patient populations, and each captures different processes in the pathology of inflammation (i.e., CAMs, chemokines, acute phase reactant), we were only able to evaluate a limited number of inflammatory mediators. Therefore, it is possible that circulating inflammatory biomarkers not represented in our panel may be more important in the pathophysiology of CAD in older adults. Moreover, the cross-sectional design of our study precludes us from directly evaluating the association between circulating biomarkers of inflammation and incident CAD, or prognosis in individuals with established CAD. In addition, circulating biomarkers of inflammation are a surrogate for the biological processes taking place at the site of the lesion, and may not capture all biological processes that are relevant to the development and progression of CAD. The absence of a significant association between higher circulating levels of seemingly established individual inflammatory biomarkers (hs-CRP, MCP-1, CAMs) and the presence and severity of obstructive CAD, and the inverse association with a less well understood inflammatory biomarker (ENA-78), highlights the complex pathophysiology of CAD development and progression in this patient population, and the need for more advanced and unbiased methods to identify key processes underlying the development and progression of obstructive CAD.

Systems biology studies have demonstrated that gene expression signatures in PBMCs are predictive of the presence and extent of coronary atherosclerosis, and gene expression changes in PBMCs mirror gene expression changes in the atherosclerotic lesion.[99, 100] In addition, PBMCs are integral mediators of vascular inflammation and express signaling molecules that drive monocyte recruitment and infiltration into the vessel (including cytokines, chemokines, and CAMs), ultimately leading to plaque development and obstructive CAD.[114] Therefore, we took a systems biology approach to characterize the association between global gene expression changes in PBMCs and the presence and severity of CAD. Our global gene expression analysis revealed 512 genes that were significantly correlated with CAD severity. The observed differential regulation of gene expression in PBMCs is consistent with a previous report of individuals undergoing coronary angiography (age range 42-82 years) that identified 526 genes with at least 1.3-fold differential expression in 27 CAD cases (defined as ≥70% stenosis in one or ≥50% stenosis in two major coronary vessels) compared to 14 controls (defined as 0% stenosis in all vessels).[99] Our findings indicate that PBMC gene expression in older individuals is dysregulated in the presence of obstructive CAD, and may provide insight into the key mechanisms underlying the development and progression of CAD.

Functional annotation of genes dysregulated in the presence of obstructive CAD revealed that the most highly enriched annotation cluster included genes key in the regulation of inflammation. Interestingly, multiple genes that were positively correlated with CAD score in our global analysis have been implicated in the nuclear factor-kappa B (NF-κB) mediated inflammatory response; NF-κB is the central transcriptional regulator of vascular inflammation, and has previously been implicated in CAD.[115] For example, *CHUK* and *PRDX3* are integral to the activation of NF-κB.[116, 117] Similarly, *JAK2* is activated by NF-κB responsive genes,[118] and NF-κB has been shown to regulate *FAS* mediated apoptosis.[119] The association between key genes involved in the innate-immune mediated inflammatory

response and CAD is consistent with a previous study in individuals <65 years of age that identified 160 genes that correlated with CAD severity, many of which were involved in the inflammatory response and apoptosis;[100] however, we are the first to utilize global gene expression analyses to identify key biological processes that are enriched in the development and progression of CAD in older individuals.

Similarly, in their development of a gene expression algorithm for assessment of obstructive CAD, Elashoff, et. al. identified 655 genes that were associated with the presence of CAD in both the CATHGEN registry and the PREDICT study, which reflected the processes of inflammation, immune cell differentiation, and apoptosis.[104] In order to more directly compare gene ontologies from genes identified in the CATHGEN/PREDICT analysis to the SAMARA study, we repeated our DAVID analysis using the 655 overlapping genes from the CATHGEN and PREDICT analyses, which similarly identified genes involved in inflammation and apoptosis as the most highly enriched biological processes. Genes identified in both analyses include *CASP4*, which has been implicated in both inflammation and apoptosis,[120] *HLA-E*, which regulates endothelial activation[121] and *MAPK14*, a member of the mitogen-activated protein kinase family, which has previously been implicated in inflammation and CVD.[122] Our findings indicate that expression of these genes in PBMCs, all of which are involved in the inflammatory response, is consistently associated with the presence CAD. Future studies are warranted to define their direct functional role in the pathogenesis and progression of CAD, and to rigorously evaluate the role of upstream pathways that regulate inflammation signaling, and thus CAD development and progression.

Our global gene expression analysis is limited by the use of PBMC RNA expression as a surrogate for capturing biological processes taking place at the site of the atherosclerotic lesion. Although this is a limitation of our analysis, PBMC gene expression has been shown to reflect atherosclerotic lesion gene expression, and the relative ease of obtaining PBMC samples allowed us to recruit a large study cohort to conduct the analysis. Importantly,

microarray technologies are vulnerable to false-positive findings, and validation of key genes by quantitative real time-polymerase chain reaction (qRT-PCR), and replication in an independent cohort remain necessary. Similarly, functional annotation tools such as DAVID cluster genes into functional groups based on their association with multiple biological processes, allowing a more insightful biological interpretation of large gene lists; however, the results from such analyses can be impacted by multiple factors and must be interpreted cautiously. Lastly, although our gene expression analysis took a non-biased global approach to identify the key biological processes underlying the development and progression of CAD, such systems biology approaches are limited to pre-defined pathways and biological processes. Therefore, follow-up studies using candidate pathway approaches provide the means to rigorously characterize upstream pathways hypothesized to be important regulators of systemic and vascular inflammation, and evaluate their potential utility as therapeutic targets.

Conclusions

In summary, our analysis of circulating inflammatory mediators did not demonstrate that plasma levels of established systemic or vascular inflammatory biomarkers are elevated in older patients with obstructive CAD. However, a functional analysis of genes associated with the presence and severity of obstructive CAD reflected differential expression of genes regulating multiple processes. Most notably, key genes regulating the inflammatory response were associated with the presence and severity of CAD. Future studies remain necessary to identify the key upstream pathways regulating the development and progression of obstructive CAD and advanced inflammation in this population, and ultimately evaluate the efficacy of novel anti-inflammatory therapeutic strategies that modulate these pathways.

Tables

Table 2.1. Study population characteristics by obstructive CAD status.

Characteristic	Obstructive CAD	No Obstructive CAD	P
N	93	50	
Age (years)	74±6.3	73±6.2	0.220
Male gender (%)	57 (61%)	18 (36%)	0.004
Caucasian (%)	74 (80%)	37 (74%)	0.446
Body mass index (kg/m^2)	29±6.3	30±7.1	0.323
Current smoker (%)	4 (4%)	3 (6%)	0.653
Diabetes (%)	38 (41%)	17 (34%)	0.421
Hyperlipidemia (%)	74 (80%)	28 (56%)	0.003
Hypertension (%)	82 (88%)	44 (88%)	0.976
Previous myocardial infarction (%)	41 (44%)	3 (6%)	<0.001
Multivessel disease (%)	62 (67%)	0 (0%)	<0.001
Systolic blood pressure (mmHg)	143±20	141±20	0.573
Diastolic blood pressure (mmHg)	77±13	78±12	0.588
Statin use (%)	74 (80%)	23 (46%)	<0.001
Aspirin use (%)	78 (84%)	33 (66%)	0.015
Clopidogrel use (%)	33 (36%)	6 (12%)	0.003

Data presented as mean ± standard deviation or count (proportion).

Age, body mass index, and diastolic blood pressure were not normally distributed and were log-transformed prior to analysis.

Table 2.2. Association between plasma inflammatory biomarker levels and CAD score.

Analyte	Parameter Estimate	Standard Error	Partial R^2	P
hsCRP*				
Unadjusted	0.095	0.068	0.014	0.164
Adjusted	0.133	0.077	0.022	0.088
MCP-1				
Unadjusted	-0.010	0.031	0.001	0.748
Adjusted	-0.015	0.035	0.001	0.673
ENA-78				
Unadjusted	-0.145	0.046	0.065	0.002
Adjusted	-0.114	0.052	0.035	0.029
CAM Score				
Unadjusted	0.145	0.131	0.009	0.270
Adjusted	0.104	0.151	0.003	0.495

Following log-transformation, associations between each analyte and CAD score were evaluated by regression.

Adjusted model: adjusted for age, race, gender, obesity, diabetes mellitus, hyperlipidemia, statin use.

*53 (37%) of values were above the limit of detection and imputed to a value of 15.2 mg/L (1.5-fold higher than the highest standard).

Table 2.3. Comparison of plasma inflammatory biomarker levels between subjects with obstructive CAD and subjects without obstructive CAD.

Analyte	Obstructive CAD	No Obstructive CAD	Unadjusted P	Adjusted P*
hsCRP^ (mg/L)	5.7 (13)	4.6 (14)	0.198	0.108
MCP-1 (pg/mL)	122 (57)	115 (46)	0.663	0.512
ENA-78 (pg/mL)	998 (923)	1638 (1380)	0.002	0.028
CAM Score	0.10 (2.6)	-0.07 (2.8)	0.489	0.748

Data presented as median (interquartile range). Following log-transformation, plasma levels of each analyte were compared by regression.

*Adjusted model: adjusted for age, race, gender, obesity, diabetes mellitus, hyperlipidemia, statin use.

^53 (37%) of values were above the limit of detection and imputed to a value of 15.2 mg/L (1.5-fold higher than the highest standard).

Table 2.4. Proportion of subjects with high (>median) or low (≤median) levels of inflammatory biomarkers by obstructive CAD status.

Analyte	Obstructive CAD	No Obstructive CAD	P
hsCRP	49 (53%)	22 (44%)	0.322
hsCRP >3 mg/L	71 (76%)	32 (64%)	0.117
MCP-1	49 (53%)	22 (44%)	0.322
ENA-78	39 (42%)	32 (64%)	0.012
CAM Score	47 (51%)	24 (48%)	0.772

Data presented as N (%) above median in each group.

Chi-square p-value is presented.

Table 2.5. Functional classification of genes that associated with the severity of CAD in SAMARA.

Annotation Cluster	Representative Annotation Terms	Genes	Fold Enrichment	P
1	Immune System Development	20	2.6	0.0006
2	Antigen Processing	9	10.0	0.0007
3	Positive Regulation Protein Processing	17	2.6	0.0019
4	Actin Related	6	7.3	0.0024
5	Negative Regulation of Transcription	8	5.0	0.0025
6	Ras GTPase	13	3.0	0.0032
7	Ubiquitin Regulation	13	3.8	0.0046
8	Regulation of DNA Binding	12	3.1	0.0047
9	Antigen Presentation	9	9.0	0.0058
10	RNA Splicing	21	2.1	0.0081

The 10 functional annotation clusters with the highest enrichment scores are presented in rank order.

“Representative Annotation Terms” provides a description of each functional cluster.

“Genes” indicates the number of genes from the SAMARA gene list that were included in each annotation cluster.

“Fold enrichment” indicates the number of genes in each functional annotation cluster relative to the number that would be expected by chance.

The p-value for each annotation cluster is provided.

Table 2.6. Functional classification of genes differentially expressed in CAD patients in the CATHGEN and PREDICT studies.

Annotation Cluster	Representative Annotation Terms	Genes	Fold Enrichment	P
1	Ribosome Component	20	4.56	<0.0001
2	Regulation of Apoptosis	54	1.94	<0.0001
3	Positive Regulation of Apoptosis	35	2.43	<0.0001
4	Immune Activation	25	2.84	<0.0001
5	Immune Differentiation	20	2.89	0.0004
6	Negative Regulation of Apoptosis	25	2.04	0.0013
7	Positive Immune Regulation	15	2.95	0.0016
8	Immune Proliferation	10	3.49	0.0022
9	Histone H2A	5	8.25	0.0026
10	Phosphorylation	50	1.56	0.0035

The 10 functional annotation clusters with the highest enrichment scores are presented in rank order.

“Representative Annotation Terms” provides a description of each functional cluster.

“Genes” indicates the number of genes from the SAMARA gene list that were included in each annotation cluster.

“Fold enrichment” indicates the number of genes in each functional annotation cluster relative to the number that would be expected by chance.

The p-value for each annotation cluster is provided.

Figures

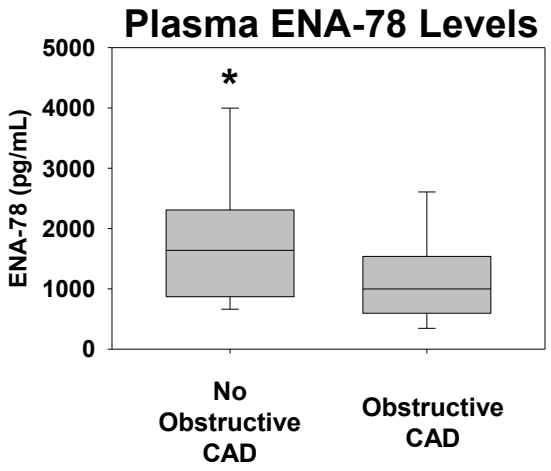

Figure 2.1. Plasma ENA-78 levels by obstructive CAD status.

The distribution of plasma ENA-78 levels is displayed in subjects with (n=93) and without (n=50) obstructive CAD. The horizontal line within the box represents the median, the edges of the box represent the 25th and 75th percentiles, and the error bars represent the 10th and 90th percentiles. *P<0.05 compared to subjects with obstructive CAD in both the unadjusted and adjusted models.

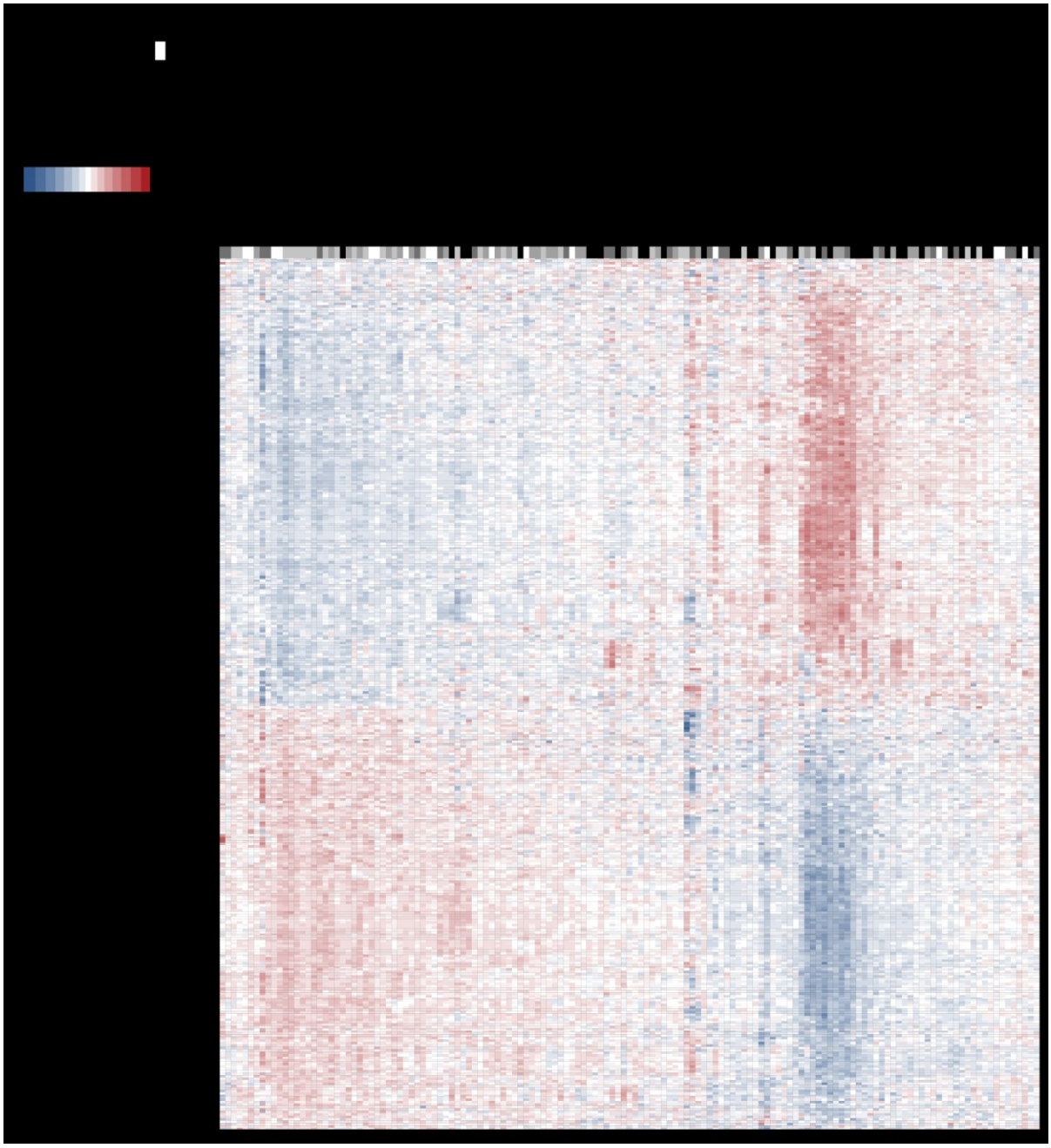

Figure 2.2. Heat-map displaying the 708 probes that were significantly correlated with CAD score with absolute correlation coefficient (r_s) of ≥0.25.

Each column represents one subject; the first row displays the CAD score; darker boxes represent more severe CAD. Individual gene expression profiles are represented in rows, red indicates a positive correlation with CAD score and blue indicates an inverse association with CAD score with the color scale designating the relative expression (log_2) of each gene.

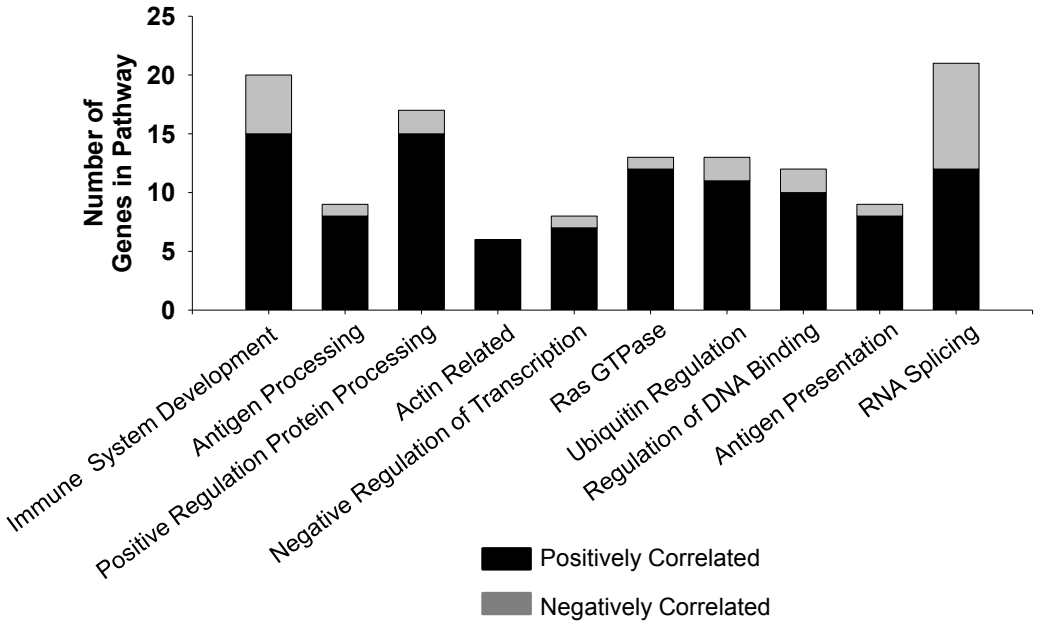

Figure 2.3. Number of genes from each of the top 10 functional annotation clusters identified by DAVID.

The number of genes from each of the top 10 functional annotation clusters identified by DAVID is displayed. The number of genes positively correlated with the CAD score are represented in black and the number of genes negatively correlated with the CAD score are represented in gray. Overall, 67 (75%) of genes in these pathways were positively correlated with the CAD score.

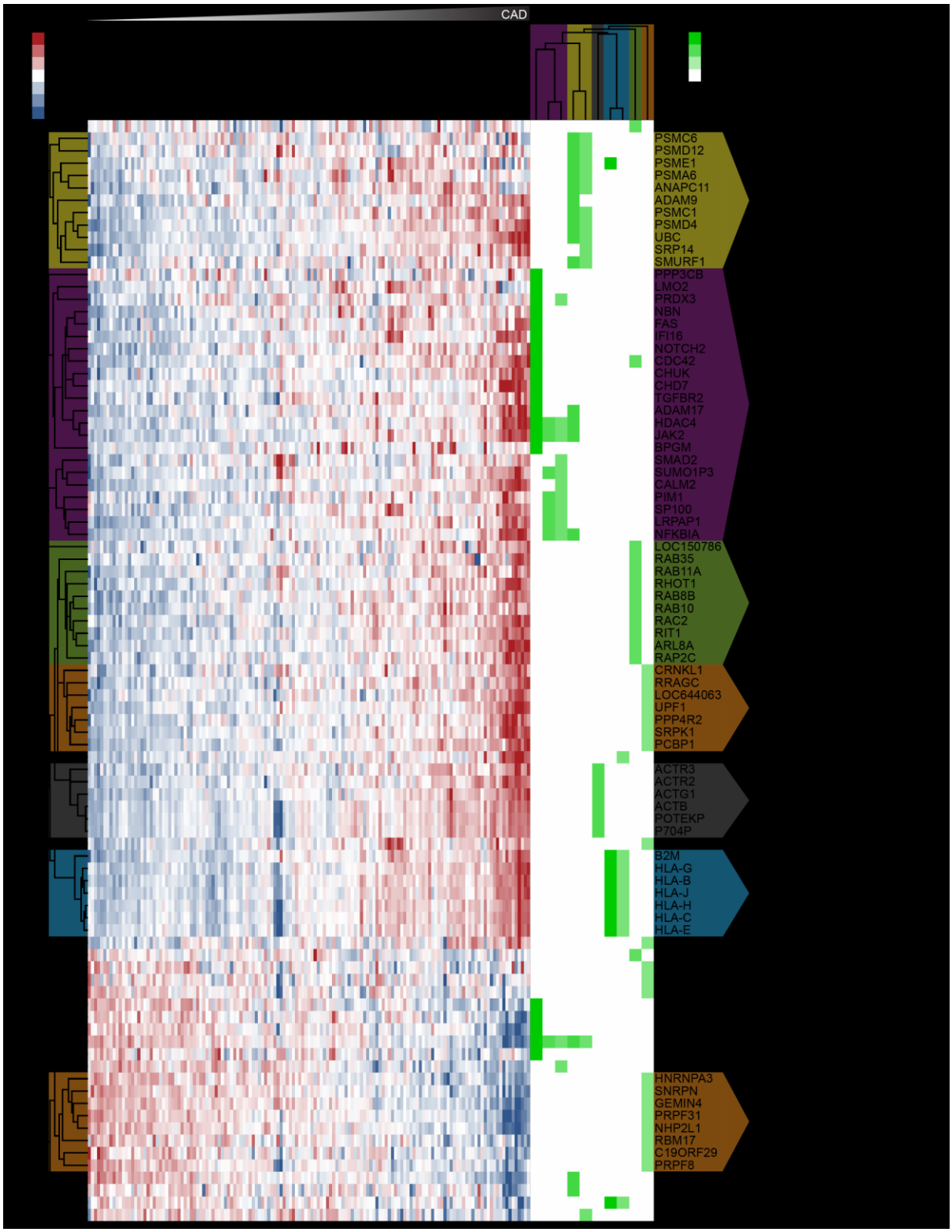
CAD
PSMC6
PSMD12
PSME1
PSMA6
ANAPC11
ADAM9
PSMC1
PSMD4
UBC
SRP14
SMURF1
PPP3CB
LMO2
PRDX3
NBN
FAS
IFI16
NOTCH2
CDC42
CHUK
CHD7
TGFBR2
ADAM17
HDAC4
JAK2
BPGM
SMAD2
SUMO1P3
CALM2
PIM1
SP100
LRPAP1
NFKBIA
LOC150786
RAB35
RAB11A
RHOT1
RAB8B
RAB10
RAC2
RIT1
ARL8A
RAP2C
CRNKL1
RRAGC
LOC644063
UPF1
PPP4R2
SRPK1
PCBP1
ACTR3
ACTR2
ACTG1
ACTB
POTEKP
P704P
B2M
HLA-G
HLA-B
HLA-J
HLA-H
HLA-C
HLA-E
HNRNPA3
SNRPN
GEMIN4
PRPF31
NHP2L1
RBM17
C19ORF29
PRPF8

Figure 2.4. (Preceding page) Heat map displaying genes correlated with presence and severity of CAD that were represented in the top 10 functional pathways from DAVID. The red-blue heat map (left) represents the relative expression (log_2) of a subset of microarray probes (rows) identified in the top 10 categories of pathway enrichment by DAVID analysis. As shown in Figure 2, the gene expression of subjects (columns) clustered generally according to CAD classification, increasing from left to right. The green heat map (middle) indicates the DAVID category of each probe and is represented by the fold-enrichment ($antilog_2$) of the functional category. The functional clusters of gene expression are indicated by color (right).

Gene Lists

Gene List 1. List of 512 unique genes that correlate with CAD score with absolute correlation coefficient (r_s) of ≥0.25

Gene Symbol	r	p-value(correlation)
PHOSPHO2	-0.380723	2.72E-06
LOC100287628	-0.370113	5.37E-06
NSUN5P1	-0.363053	8.33E-06
PDE8B	-0.356918	1.21E-05
PMS2L1	-0.341573	2.98E-05
LRDD	-0.341141	3.05E-05
AZI1	-0.330346	5.59E-05
AGK	-0.32976	5.77E-05
PMS2	-0.328076	6.33E-05
LOC100289173	-0.326178	7.02E-05
SSNA1	-0.320579	9.48E-05
DMAP1	-0.318288	0.000107
LRRC56	-0.316948	0.000115
PDDC1	-0.31586	0.000122
RG9MTD3	-0.315461	0.000124
FAM128B	-0.314821	0.000128
BCL7A	-0.312254	0.000147
NSUN5	-0.309186	0.000172
FIS1	-0.307483	0.000187
APBB1	-0.306803	0.000194
SNHG10	-0.306109	0.000201
SARM1	-0.305626	0.000206
SPG7	-0.303997	0.000223
TCTN1	-0.30393	0.000224
PEMT	-0.30294	0.000235
LOC642346	-0.302004	0.000247
CHD1L	-0.301706	0.00025
PI4KA	-0.300811	0.000262
NOSIP	-0.300801	0.000262
EIF3L	-0.300093	0.000271
ZCCHC7	-0.299291	0.000282
IPO5	-0.298768	0.000289
LRRC58	-0.297993	0.000301
POP5	-0.297802	0.000304
BTF3	-0.297781	0.000304
RBM19	-0.297768	0.000304

DNAJA3	-0.296821	0.000318
PMS2L11	-0.293494	0.000374
ICAM4	-0.293459	0.000375
KIAA1407	-0.293196	0.00038
CHD9	-0.292245	0.000398
ACOT1	-0.29185	0.000405
EFNB1	-0.290846	0.000425
SEC31B	-0.290756	0.000427
RFTN1	-0.290151	0.000439
RPAIN	-0.289555	0.000452
PAOX	-0.289307	0.000457
HIST1H4C	-0.28925	0.000459
TRAIP	-0.289019	0.000464
DNAJC27	-0.288904	0.000466
C11ORF2	-0.288868	0.000467
RPL3	-0.287526	0.000498
RSAD1	-0.287234	0.000505
SEPT6	-0.286738	0.000517
NELF	-0.286699	0.000518
ESF1	-0.286549	0.000521
RPL12P6	-0.286071	0.000533
XPNPEP1	-0.28564	0.000544
MRPL10	-0.285592	0.000545
ELAC2	-0.285449	0.000549
STAG3L2	-0.284399	0.000576
EXOSC7	-0.284302	0.000579
ASXL1	-0.284097	0.000585
C13ORF15	-0.283059	0.000614
COQ4	-0.282866	0.000619
WDR92	-0.281829	0.00065
KIAA1191	-0.281759	0.000652
MTF2	-0.281753	0.000652
HARS	-0.281648	0.000655
DIDO1	-0.281631	0.000656
NKTR	-0.281499	0.00066
MCM7	-0.280695	0.000685
SUSD3	-0.280348	0.000696
LOC152217	-0.280014	0.000706
HNRNPA3	-0.279411	0.000726
LOC100287580	-0.279155	0.000735
WDR59	-0.278758	0.000748

SLC22A23	-0.278242	0.000766
EEF1D	-0.277013	0.00081
GLTSCR2	-0.276717	0.000821
HSD17B10	-0.276713	0.000821
ZNF544	-0.276509	0.000829
QRSL1	-0.276184	0.000841
PNPLA4	-0.275892	0.000852
ABI2	-0.275654	0.000862
LOC400987	-0.274663	0.000901
RPL34	-0.274657	0.000901
TRMU	-0.274516	0.000907
TAF1C	-0.274331	0.000915
ZCCHC17	-0.273676	0.000942
SCARNA15	-0.272891	0.000976
LOC100128355	-0.272884	0.000976
ZNF783	-0.272664	0.000986
IMP4	-0.272406	0.000997
RPSAP52	-0.272204	0.001006
SAMM50	-0.271713	0.001028
OGFOD1	-0.271353	0.001045
NEURL4	-0.271015	0.001061
GTF2H4	-0.270961	0.001063
CEBPZ	-0.27052	0.001084
NDRG2	-0.270158	0.001102
BCL11A	-0.269978	0.001111
FBL	-0.269814	0.001119
TRAPPC6A	-0.269654	0.001127
LOC648771	-0.269333	0.001143
CHAF1A	-0.268751	0.001172
MYO19	-0.268688	0.001176
PYGO2	-0.268632	0.001179
RBM17	-0.268516	0.001185
SEC63	-0.268478	0.001187
BAT3	-0.268405	0.00119
ACOT2	-0.268359	0.001193
ATF7IP2	-0.268134	0.001205
DDX51	-0.267958	0.001214
MARCH9	-0.267878	0.001218
EIF3D	-0.267338	0.001247
METTL2B	-0.267136	0.001259
FAM160B2	-0.267065	0.001262

ZBTB5	-0.266542	0.001292
RSL1D1	-0.265307	0.001363
PMPCA	-0.264789	0.001394
HADH	-0.264713	0.001399
TRPT1	-0.264425	0.001416
LOC100132364	-0.264402	0.001418
LRPPRC	-0.264373	0.001419
PPIAL3	-0.264058	0.001439
PET112L	-0.263928	0.001447
PRPF31	-0.263926	0.001447
CD79A	-0.263663	0.001464
AKAP1	-0.263594	0.001468
RRP1B	-0.263268	0.001489
C18ORF22	-0.263203	0.001493
TMEM223	-0.262947	0.00151
ZG16B	-0.262938	0.00151
FAM195A	-0.262875	0.001514
ZNHIT2	-0.262821	0.001518
HAX1	-0.262812	0.001518
AFG3L1	-0.262521	0.001538
SMYD3	-0.261951	0.001576
TRIB2	-0.261838	0.001583
GCDH	-0.261632	0.001597
PCCA	-0.261607	0.001599
DHX30	-0.26083	0.001653
EIF1AX	-0.260553	0.001673
TTC17	-0.260116	0.001704
SNRPN	-0.260089	0.001706
PRPF8	-0.259952	0.001716
SGSM3	-0.25957	0.001744
ZNF10	-0.259431	0.001755
LNPEP	-0.259146	0.001776
MBD3	-0.259146	0.001776
UBE2Q2P1	-0.259144	0.001776
MUTYH	-0.258747	0.001807
NHP2L1	-0.258659	0.001813
SFRS14	-0.258545	0.001822
CXORF57	-0.258446	0.00183
ZIK1	-0.258253	0.001845
RPL15P18	-0.257888	0.001873
LOC442454	-0.257837	0.001877

LOC399804	-0.257793	0.001881
THEM4	-0.257682	0.00189
SHMT2	-0.257594	0.001897
RBM25	-0.257522	0.001903
UTP14A	-0.25735	0.001916
ZNF91	-0.257312	0.001919
MRPS2	-0.257241	0.001925
PMEPA1	-0.257067	0.001939
ZNF439	-0.256951	0.001949
DGCR6L	-0.25689	0.001954
TMC8	-0.256831	0.001959
DDT	-0.256806	0.001961
IL11RA	-0.256638	0.001975
EVL	-0.256122	0.002018
ZNF500	-0.256096	0.00202
ING5	-0.255857	0.002041
LOC283663	-0.255704	0.002054
C19ORF29	-0.255674	0.002056
ELP2	-0.255492	0.002072
XRCC1	-0.255116	0.002105
NDUFV1	-0.254893	0.002125
EGFL8	-0.254582	0.002152
ANKRD36	-0.254482	0.002161
RPS10	-0.254454	0.002164
CC2D2B	-0.254368	0.002172
ZNF335	-0.254316	0.002176
DDX27	-0.254213	0.002186
FLJ10038	-0.254057	0.0022
P4HTM	-0.253959	0.002209
LOC338799	-0.253875	0.002217
NCAPH2	-0.253616	0.002241
ZNF571	-0.253532	0.002249
C5ORF45	-0.253488	0.002253
WDR74	-0.253383	0.002263
GRASP	-0.253314	0.002269
FAM82B	-0.252999	0.002299
RPS15AP12	-0.252875	0.002311
GEMIN4	-0.252438	0.002353
WDR54	-0.252306	0.002366
ZNF232	-0.252203	0.002376
ITM2C	-0.251943	0.002402

SETDB2	-0.251806	0.002415
MRPL41	-0.251651	0.002431
ALG10B	-0.251628	0.002433
DYRK4	-0.251569	0.002439
C12ORF65	-0.25134	0.002462
FCRLA	-0.251252	0.002471
LOC389901	-0.25114	0.002482
PRMT1	-0.251094	0.002487
ADAT2	-0.25079	0.002518
PRKAB2	-0.250731	0.002525
CD1C	-0.250706	0.002527
CLN8	-0.250697	0.002528
GPATCH1	-0.250685	0.002529
ZNF446	-0.250622	0.002536
WDR73	-0.25055	0.002543
INTS10	-0.25022	0.002578
RBM42	-0.250105	0.00259
R3HDM2	0.250157	0.002585
IER2	0.250739	0.002524
NFE2	0.250767	0.002521
PSMA6	0.250903	0.002507
RAC2	0.250928	0.002504
CREG1	0.250932	0.002504
FCHSD2	0.250977	0.002499
KPNA4	0.251151	0.002481
NMI	0.251155	0.002481
CMTM3	0.251184	0.002478
MKRN1	0.251434	0.002452
RAB10	0.251523	0.002444
KIAA0240	0.251611	0.002435
CNIH4	0.251733	0.002422
ARNT	0.251745	0.002421
RAB11A	0.251959	0.0024
SQRDL	0.252258	0.002371
FTLP2	0.252535	0.002344
LIMD2	0.252879	0.00231
OAZ1	0.252913	0.002307
GRB10	0.252957	0.002303
C6ORF150	0.252969	0.002302
SMURF1	0.252997	0.002299
TMEM167A	0.253171	0.002283

SLC39A11	0.253272	0.002273
RGS19	0.25345	0.002256
ME2	0.25407	0.002199
MPP1	0.254215	0.002186
LCP1	0.254253	0.002182
ASTN2	0.254274	0.00218
IRF9	0.254303	0.002178
MYL6	0.254318	0.002176
HLA-H	0.254332	0.002175
PGK1	0.254332	0.002175
LPCAT2	0.254622	0.002149
HARBI1	0.254643	0.002147
FLI1	0.254658	0.002146
PLBD1	0.254881	0.002126
ARF6	0.254912	0.002123
COTL1	0.25496	0.002119
ARPC3	0.255204	0.002097
CAPZA1	0.255374	0.002082
C16ORF70	0.255794	0.002046
CD274	0.255853	0.002041
GMFG	0.25591	0.002036
LOC150786	0.256099	0.00202
SLK	0.256227	0.002009
CTSA	0.256229	0.002009
UBC	0.256489	0.001987
SLC44A1	0.256493	0.001987
TALDO1	0.256684	0.001971
ATF7	0.256808	0.001961
ADM	0.256827	0.001959
MGC4473	0.256899	0.001953
WDR20	0.256983	0.001946
RNF10	0.256985	0.001946
CHD7	0.257088	0.001938
LAMP1	0.257447	0.001909
C4ORF34	0.25805	0.001861
CALM2	0.258764	0.001805
ZNF24	0.258789	0.001803
HLA-E	0.25894	0.001792
BPGM	0.25899	0.001788
LMO2	0.259079	0.001781
JAK2	0.25915	0.001776

TTRAP	0.259196	0.001772
AIF1	0.259236	0.001769
NOTCH2	0.259524	0.001748
KRCC1	0.25961	0.001742
SLC38A2	0.259618	0.001741
ATP5L	0.259849	0.001724
GOLGA7	0.2599	0.00172
C1ORF63	0.259967	0.001715
ERBB2IP	0.259997	0.001713
FAM100B	0.260166	0.001701
C20ORF24	0.260219	0.001697
PPP4R2	0.260282	0.001692
TPM3	0.260681	0.001664
UPF1	0.260759	0.001658
RRAGC	0.260763	0.001658
DEGS1	0.260872	0.00165
CRADD	0.260985	0.001642
YWHAZ	0.261023	0.00164
ATP11B	0.26103	0.001639
PCBP1	0.26104	0.001638
TMEM140	0.261198	0.001627
FAU	0.261229	0.001625
UBE2D3P	0.261414	0.001612
VCPIP1	0.261735	0.00159
S100P	0.261949	0.001576
MCL1	0.261972	0.001574
HLA-C	0.262117	0.001565
MAFG	0.26225	0.001556
FAM104A	0.262256	0.001555
YOD1	0.262542	0.001536
GSR	0.262571	0.001534
LOC647252	0.262707	0.001525
ERO1L	0.262756	0.001522
TAP1	0.262838	0.001517
SMAD2	0.263371	0.001482
LOC220594	0.263501	0.001474
CDC42SE1	0.264274	0.001426
TMSL1	0.26428	0.001425
LRPAP1	0.264591	0.001406
NBN	0.264633	0.001404
SAP18	0.264673	0.001401

LOC728875	0.264742	0.001397
SLC37A3	0.264791	0.001394
CD63	0.265314	0.001363
CRNKL1	0.265564	0.001348
ZNF93	0.265645	0.001343
HLA-J	0.265809	0.001334
RAB35	0.265874	0.00133
TSC22D2	0.265971	0.001324
SUMO1P3	0.266049	0.00132
THRAP3	0.266288	0.001306
SEC22B	0.266584	0.001289
SCYL2	0.266603	0.001288
PSMC6	0.266704	0.001283
TOR1AIP2	0.266889	0.001272
GLRX	0.266937	0.00127
ZC3H11A	0.266937	0.00127
CASP4	0.267019	0.001265
TGFBR2	0.267269	0.001251
SRPK1	0.2673	0.00125
FAS	0.267399	0.001244
IFI16	0.267439	0.001242
PRR13	0.267563	0.001235
LOC644063	0.267683	0.001229
TAB3	0.267731	0.001226
LOC100133398	0.267775	0.001224
NBPF14	0.267941	0.001215
ADAR	0.268058	0.001209
CNTNAP3	0.268145	0.001204
HINT3	0.268491	0.001186
ACTR3	0.268506	0.001185
TXN	0.26873	0.001173
UPP1	0.26877	0.001171
SRP14	0.26905	0.001157
ACPL2	0.269062	0.001156
C14ORF43	0.269144	0.001152
TXNIP	0.269491	0.001135
CXCR4	0.269493	0.001135
PSME1	0.269583	0.00113
CD46	0.269728	0.001123
FAM27C	0.269829	0.001118
FBXO30	0.270083	0.001105

PRDX3	0.270333	0.001093
LSM14B	0.270696	0.001076
VIM	0.270828	0.00107
KPNB1	0.270847	0.001069
MIER1	0.271566	0.001035
GNB4	0.271773	0.001025
TMBIM6	0.272034	0.001014
RIT1	0.272088	0.001011
LOC644101	0.272198	0.001006
TOP1P2	0.272601	0.000988
NACC1	0.272628	0.000987
TMED5	0.27283	0.000978
KCNE1L	0.273012	0.00097
FAM199X	0.273061	0.000968
POTEKP	0.273141	0.000965
KIDINS220	0.273243	0.00096
SMCHD1	0.273254	0.00096
CENPB	0.273319	0.000957
ACTB	0.273504	0.000949
RABAC1	0.273846	0.000935
UBE2H	0.274063	0.000926
ELF4	0.274109	0.000924
SPDYA	0.274153	0.000922
PPP3CB	0.274199	0.00092
ZEB2	0.274405	0.000912
RBM7	0.274928	0.00089
LGALS8	0.275087	0.000884
MSL3	0.275522	0.000867
CLIC1	0.275593	0.000864
WIPI1	0.275785	0.000857
LCE1A	0.276242	0.000839
B4GALT1	0.276417	0.000832
LOC100294179	0.276562	0.000827
TADA3	0.276591	0.000826
SERPINB1	0.276658	0.000823
NBR1	0.276908	0.000814
AFF1	0.277406	0.000796
ARHGDIB	0.277612	0.000788
NPTN	0.277677	0.000786
SLC30A5	0.277941	0.000777
EXOC6	0.278017	0.000774

KIAA1609	0.27834	0.000763
TOR1A	0.278773	0.000748
ADAM9	0.279527	0.000722
AGFG1	0.279581	0.00072
PPP1R10	0.279676	0.000717
CYB5R3	0.279686	0.000717
P704P	0.279836	0.000712
PLEKHH3	0.279857	0.000711
ANAPC11	0.279949	0.000708
TMEM131	0.280201	0.0007
HDAC4	0.280251	0.000699
PSMD12	0.280974	0.000676
CHUK	0.281215	0.000668
C1GALT1C1	0.281272	0.000667
DISC1	0.281969	0.000645
GATAD2A	0.282106	0.000641
DEDD	0.282463	0.000631
LOC541471	0.282616	0.000626
CHIC2	0.282683	0.000624
TRIP12	0.283729	0.000595
MOBKL1B	0.284265	0.00058
CHMP5	0.284449	0.000575
SAMSN1	0.284758	0.000567
ACTR2	0.284783	0.000566
ARL6IP6	0.28479	0.000566
LYRM1	0.284794	0.000566
YIPF1	0.284951	0.000562
PSMC1	0.285193	0.000555
CDC5L	0.285348	0.000551
ZC3H4	0.285546	0.000546
RBMS1	0.285569	0.000546
ZBTB47	0.285739	0.000541
MORF4L1	0.286104	0.000532
SGMS1	0.287062	0.000509
CNBP	0.287398	0.000501
GNAI3	0.287675	0.000494
LOC730144	0.287763	0.000492
DYNLT1	0.288181	0.000483
PSMD4	0.288263	0.000481
ETS2	0.288666	0.000472
EVI2A	0.288836	0.000468

ACTG1	0.289179	0.00046
HTATIP2	0.289443	0.000455
WAC	0.289826	0.000446
MSL3L2	0.28987	0.000445
SPATA13	0.289874	0.000445
VEZF1	0.290061	0.000441
ZBTB2	0.290502	0.000432
GRN	0.291029	0.000421
GADD45A	0.29176	0.000407
SP100	0.294742	0.000352
DPYD	0.294754	0.000352
EIF1B	0.295567	0.000339
KIAA1949	0.295771	0.000335
NEDD8	0.296075	0.00033
TBC1D5	0.296399	0.000325
ADAM17	0.296686	0.000321
ELF1	0.296783	0.000319
MYL12B	0.296819	0.000319
C16ORF57	0.297249	0.000312
GYG1	0.299087	0.000285
EAF1	0.299624	0.000278
PGM2	0.300948	0.00026
MAPK14	0.30129	0.000256
S100A6	0.301584	0.000252
SNX6	0.303058	0.000234
DCUN1D1	0.303457	0.000229
TRIM21	0.304923	0.000213
RAB8B	0.307382	0.000188
TOR1AIP1	0.309659	0.000168
ITPR2	0.310984	0.000157
DENND1B	0.311007	0.000156
CDC42	0.311383	0.000153
MAP3K5	0.311429	0.000153
ATP6V1C1	0.311458	0.000153
ABR	0.311685	0.000151
HLA-G	0.312401	0.000146
RAP2C	0.313802	0.000135
SHISA5	0.314539	0.00013
NSUN3	0.314743	0.000129
UBA3	0.314829	0.000128
LOC100292967	0.315604	0.000123

DNAJA1	0.317194	0.000113
RHOT1	0.317996	0.000109
IDH1	0.318034	0.000109
NFKBIA	0.318363	0.000107
HLA-B	0.318876	0.000104
METTL9	0.3196	9.99E-05
SNX3	0.320938	9.3E-05
BRD2	0.322421	8.6E-05
PPT1	0.323086	8.29E-05
NBPF3	0.32329	8.2E-05
ZMYM5	0.323605	8.07E-05
TMEM165	0.323685	8.03E-05
UBQLN2	0.324174	7.82E-05
TMSL3	0.32476	7.58E-05
B2M	0.325663	7.22E-05
TMEM167B	0.327413	6.57E-05
RNF146	0.328173	6.3E-05
MYL12A	0.329292	5.92E-05
ARL8A	0.33201	5.1E-05
SYS1	0.33403	4.56E-05
SSFA2	0.340979	3.08E-05
MAP2K6	0.34147	3E-05
PIM1	0.34503	2.44E-05
GPER	0.3577	1.15E-05
LONRF1	0.380004	2.85E-06

Gene List 2. Genes identified in functional analysis of the SAMRA gene list.

Genes	R	P	Pathway(s)
ACTB	0.273504	0.000949	Actin related
ACTG1	0.28918	0.00046	Actin related
ACTR2	0.28478	0.00057	Actin related
ACTR3	0.26851	0.00119	Actin related
ADAM17	0.29669	0.00032	Immune system development, Positive regulation of protein processing
ADAM9	0.27953	0.00072	Positive regulation of protein processing
ADAR	0.26806	0.00121	RNA splicing
ANAPC11	0.27995	0.00071	Positive regulation of protein processing
ARF6	0.25491	0.00212	Ras GTPase
ARL8A	0.33201	5.10E-05	Ras GTPase
B2M	0.32566	7.22E-05	Antigen processing, Antigen presentation
BCL11A	-0.27	0.00111	Immune system development
BPGM	0.25899	0.00179	Immune system development
C19ORF29	-0.2557	0.00206	RNA splicing
CALM2	0.25876	0.00181	Regulation of DNA binding
CD1C	-0.2507	0.00253	Antigen processing, Antigen presentation
CD79A	-0.2637	0.00146	Immune system development
CDC42	0.31138	0.00015	Immune system development, Ras GTPase
CDC5L	0.28535	0.00055	RNA splicing
CHD7	0.25709	0.00194	Immune system development
CHUK	0.28122	0.00067	Immune system development
CLN8	-0.2507	0.00253	Ubiquitin regulation
CRADD	0.26099	0.00164	Antigen presentation
CRNKL1	0.26556	0.00135	RNA splicing
DNAJA3	-0.2968	0.00032	Immune system development, Positive regulation of protein processing, Negative regulation of transcription, Ubiquitin regulation, Regulation of DNA binding
DNAJC27	-0.2889	0.00047	Ras GTPase
FAS	0.2674	0.00124	Immune system development
GEMIN4	-0.2524	0.00235	RNA splicing
HDAC4	0.28025	0.0007	Immune system development, Positive regulation of protein processing, Negative regulation of transcription, Regulation of DNA binding
HLA-B	0.31888	0.0001	Antigen processing, Antigen presentation
HLA-C	0.26212	0.00156	Antigen processing, Antigen presentation
HLA-E	0.25894	0.00179	Antigen processing, Antigen presentation
HLA-G	0.3124	0.00015	Antigen processing, Antigen presentation

HLA-H	0.25433	0.00217	Antigen processing, Antigen presentation
HLA-J	0.26581	0.00133	Antigen processing, Antigen presentation
HNRNPA3	-0.2794	0.00073	RNA splicing
IFI16	0.26744	0.00124	Immune system development
JAK2	0.25915	0.00178	Immune system development, Positive regulation of protein processing, Negative regulation of transcription, Regulation of DNA binding
LMO2	0.25908	0.00178	Immune system development
LOC150786	0.2561	0.00202	Ras GTPase
LOC389901	-0.2511	0.00248	Immune system development
LOC399804	-0.2578	0.00188	Regulation of DNA binding
LOC644063	0.26768	0.00123	RNA splicing
LRPAP1	0.26459	0.00141	Negative regulation of transcription, Regulation of DNA binding
NBN	0.26463	0.0014	Immune system development
NFKBIA	0.31836	0.00011	Positive regulation of protein processing, Negative regulation of transcription, Regulation of DNA binding
NHP2L1	-0.2587	0.00181	RNA splicing
NOTCH2	0.25952	0.00175	Immune system development
P704P	0.27984	0.00071	Actin related
PCBP1	0.26104	0.00164	RNA splicing
PEMT	-0.3029	0.00024	Positive regulation of protein processing
PIM1	0.34503	2.44E-05	Negative regulation of transcription, Regulation of DNA binding
PMS2	-0.3281	6.3E-05	Immune system development
POTEKP	0.27314	0.00096	Actin related
PPP3CB	0.2742	0.00092	Immune system development
PPP4R2	0.26028	0.00169	RNA splicing
PRDX3	0.27033	0.00109	Immune system development
PRPF31	-0.2639	0.00145	RNA splicing
PRPF8	-0.26	0.00172	RNA splicing
PSMA6	0.2509	0.00251	Positive regulation of protein processing, Ubiquitin regulation
PSMC1	0.28519	0.00056	Positive regulation of protein processing, Ubiquitin regulation
PSMC6	0.2667	0.00128	Positive regulation of protein processing, Ubiquitin regulation
PSMD12	0.28097	0.00068	Positive regulation of protein processing, Ubiquitin regulation
PSMD4	0.28826	0.00048	Positive regulation of protein processing, Ubiquitin regulation

PSME1	0.26958	0.00113	Antigen processing, Positive regulation of protein processing, Ubiquitin regulation
RAB10	0.25152	0.00244	Ras GTPase
RAB11A	0.25196	0.0024	Ras GTPase
RAB35	0.26587	0.00133	Ras GTPase
RAB8B	0.30738	0.00019	Ras GTPase
RAC2	0.25093	0.0025	Ras GTPase
RAP2C	0.3138	0.00014	Ras GTPase
RBM17	-0.2685	0.00118	RNA splicing
RBM25	-0.2575	0.0019	RNA splicing
RHOT1	0.318	0.00011	Ras GTPase
RIT1	0.27209	0.00101	Ras GTPase
RRAGC	0.26076	0.00166	RNA splicing
SFRS14	-0.2585	0.00182	RNA splicing
SGSM3	-0.2596	0.00174	Positive regulation of protein processing
SMAD2	0.26337	0.00148	Regulation of DNA binding
SMURF1	0.253	0.0023	Positive regulation of protein processing, Ubiquitin regulation
SNRPN	-0.2601	0.00171	RNA splicing
SP100	0.29474	0.00035	Negative regulation of transcription, Regulation of DNA binding
SRP14	0.26905	0.00116	Ubiquitin regulation
SRPK1	0.2673	0.00125	RNA splicing
SUMO1P3	0.26605	0.00132	Negative regulation of transcription, Regulation of DNA binding
TGFBR2	0.26727	0.00125	Immune system development
TRPT1	-0.2644	0.00142	RNA splicing
UBC	0.25649	0.00199	Positive regulation of protein processing, Ubiquitin regulation
UPF1	0.26076	0.00166	RNA splicing

Gene List 3. Overlap of genes identified in the CATHEN/PREDICT analysis and the SAMARA analysis.

ACTB	MARCH9
APBB1	MCL1
B2M	METTL9
BCL7A	MYL6
C11orf2	NDRG2
C16orf57	NMI
C20orf24	PPP4R2
CALM3	RAC2
CASP4	RIT1
CLIC1	RPL34
DYNLT1	RPS10
EIF1AX	S100P
EXOC6	SAMSN1
FAU	SEC22B
GLRX	SERPINB1
GMFG	SLC37A3
GRB10	SP100
GYG1	SRPK1
HDAC4	TALDO1
HLA-E	TXN
HTATIP2	UBC
ITM2C	YOD1
MAPK14	

CHAPTER III

EVALUATION OF ARACHIDONIC ACID METABOLISM GENE EXPRESSION IN HUMANS WITH OBSTRUCTIVE CORONARY ARTERY DISEASE

Introduction

Vascular inflammation is integral to the development and progression of coronary artery disease (CAD), and activation of the inflammatory cascade contributes to plaque instability and rupture, resulting in acute coronary syndrome (ACS) events.[2 4] In addition, CAD patients with advanced vascular inflammation have poor disease prognosis and have higher risk of all-cause mortality.[12, 13] Peripheral blood mononuclear cells (PBMCs) are integral mediators of vascular inflammation, and infiltration of monocytes into the vessel wall is a key event in the pathogenesis of atherosclerosis. PBMCs express numerous signaling molecules regulating interactions between the vessel wall and circulating leukocytes, and drive monocyte recruitment and infiltration into the vessel, ultimately leading to plaque development and obstructive CAD.[114] Moreover, the presence of obstructive CAD is associated with higher risk of cardiovascular events and poor prognosis in CAD patients with advanced age.[1] Therefore, identification of biological pathways that are dysregulated in the presence of obstructive CAD in older individuals may provide insight into pathogenesis and progression of atherosclerosis, and facilitate the development of targeted therapeutic strategies for this high-risk population subset.

Arachidonic acid derived eicosanoids regulate key processes in the pathophysiology of atherosclerosis including inflammation, platelet aggregation and vascular tone.[34, 39] Genetic epidemiology studies indicate the cytochrome P450 (CYP), cyclooxygenase (COX), and

lipoxygenase (LOX) pathways of arachidonic acid metabolism are associated with coronary artery disease (CAD) risk.[48, 80, 123] For example, a polymorphism in *EPHX2* (K55R) is associated with higher epoxide hydrolase activity *in vivo* and higher risk of CAD[81] and endothelial dysfunction[82] in Caucasians. In addition, a *CYP2J2* promoter polymorphism (*CYP2J2 -50G>T*) is associated with lower *CYP2J2* promoter activity *in vitro* and higher risk of CAD in humans.[83] Similarly, the *CYP2C8* K399R variant allele has lower CYP epoxygenase activity *in vitro*[84] and is associated with higher risk of acute myocardial infarction (MI) in humans.[85] In the COX pathway, single nucleotide polymorphisms in *TBXAS1* and *PTGIS* have been associated with risk for MI and ischemic stroke and common polymorphisms in *PTGS2* have been associated with risk for ACS events.[124, 125] Similarly, in the LOX pathway a variable nucleotide tandem repeat polymorphism in the *ALOX5* promoter has been associated with the development of subclinical atherosclerosis[60] and certain haplotypes in *ALOX5AP* and *LTA4H* have been associated with risk of MI.[126, 127]

Collectively, these studies demonstrate that inter-individual variation in the arachidonic acid metabolism pathway is an important predictor of cardiovascular disease (CVD) risk. Moreover, PBMCs express numerous genes that contribute to the metabolism of arachidonic acid to biologically active eicosanoids, including components of the CYP, COX, and LOX pathways, suggesting that dysregulation of arachidonic acid metabolism may regulate monocyte activation and infiltration into the vessel wall.[99, 128] Since inflammation and monocyte infiltration into the vessel wall are key events in the pathogenesis and progression of atherosclerosis, and the CYP, COX and LOX pathways are known to regulate systemic and vascular inflammation in preclinical models, we hypothesize that dysregulation of arachidonic acid metabolism is a key driver of inflammation and the subsequent development and progression of atherosclerosis in humans. However, the contribution of inter-individual variation in the expression of genes that metabolize arachidonic acid to biologically active eicosanoids to CAD risk remains unknown. Therefore,

the objective of this analysis is to determine if genes in the arachidonic acid metabolism pathway are differentially expressed in PBMCs of older individuals with obstructive CAD, a population at high-risk for CAD clinical events.

Methods

Study Population

The Supporting A Multidisciplinary Approach to Researching Atherosclerosis (SAMARA) study enrolled consecutive patients who were at least 65 years of age and undergoing diagnostic cardiac catheterization as part of their routine care. Between March 2007 and February 2009 we enrolled 143 individuals from the University of North Carolina (UNC) Cardiac Catheterization Laboratory. Subjects with HIV or HCV infection, lymphoma, leukemia, anemia, history of rheumatoid arthritis or lupus, history of solid organ transplantation and individuals undergoing current cancer treatment or chronic immunosuppressive therapy were not eligible to participate. This study was approved by the UNC Institutional Review Board and conducted according to institutional guidelines; all participants provided written informed consent.

Sample Collection and Processing

All catheterizations took place in the morning following an overnight fast; at the start of the procedure 30 mL of blood was drawn from the femoral artery into Na-EDTA Vacutainer tubes (Becton, Dickinson and Co., Cockeysville, MD). Following blood collection RNA was isolated from PBMCs as described.[101] RNA was purified using the RNeasy mini-kit (Qiagen, Valencia, CA). RNA quantity, purity and integrity were assessed by spectrophotometry and microcapillary electrophoresis on an Agilent BioAnalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). Only RNA with an A260/280 ratio of ≥ 1.8, and an electrophoretic profile consistent with only minimal degradation was considered acceptable for use. An additional 8

mL of blood collected in tubes containing EDTA and proteinase inhibitor was processed to plasma, aliquoted, and stored at -80ºC pending analysis.

CAD Severity

Subjects underwent diagnostic left-heart catheterization as part of their routine medical care. Following the procedure, coronary angiography was utilized to classify subjects as obstructive CAD patients (≥70% stenosis in ≥1 major epicardial coronary artery); individuals with <70% occlusion in any vessel were considered free of obstructive CAD and were utilized as the comparator group.

Gene Expression Microarray

RNA was co-hybridized to Agilent G4112A Whole Human Genome 44K oligonucleotide arrays in the presence of Cyanine-3 labeled Universal Human Reference RNA (Stratagene, LaJolla, CA) for array normalization, as described.[101] Slides were hybridized and washed, then scanned on an Axon 4000b microarray scanner; data were processed using Agilent software. Only probes flagged "detected" in at least 85% of all samples were included, resulting in 18,411 probes in the final dataset. In addition, only samples with at least 90% of probes flagged detected were included in the analyses. Missing data were imputed using the k-nearest neighbor algorithm (k=10). MIAME-compliant datasets were deposited with the Gene Expression Omnibus of the National Center for Biotechnology Information (GEO Series accession number GSE12959).

Microarray expression data was corrected to account for scan-date batch effects using the remove batch-effect feature in Partek Genomics Suite v6.6. Unsupervised analyses determined the primary signal contributing to gene expression was gender. In addition, diabetes was found to be a potential confounder to CAD severity. Therefore we conditioned

the dataset using an analysis of variance (ANOVA) linear model to account for gender and diabetes.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) is a powerful analytical tool for the detection of gene expression differences in coordinated biological processes, such as metabolic signaling pathways.[129, 130] Therefore, GSEA (Version 2.0) was used to determine if the arachidonic acid metabolism pathway is "enriched" in individuals with obstructive CAD compared to individuals without obstructive CAD.

GSEA ranks a preselected list of genes according to their correlation with the phenotype being tested then calculates an enrichment score that represents the degree to which the candidate gene list is overrepresented at the top or bottom of all genes on the microarray. Significance (p-value) is determined by comparing the enrichment score of the candidate gene list to the null distribution, which is obtained from a series of random phenotype permutations. The leading-edge or "core enrichment" subset is defined as the gene(s) that appear at or before the maximum deviation of the gene set from zero, and thus is the core subset of candidate genes that accounts for the observed enrichment signal relative to global gene expression.

Genes involved in the metabolism of arachidonic acid to biologically active eicosanoids were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG)[131] 'arachidonic acid metabolism pathway' map00590, and limited to genes encoding proteins involved in arachidonic acid liberation from the cell membrane and metabolism (Table 3.1). Of the 43 genes in the arachidonic acid metabolism pathway, 40 (93%) were represented on the microarray, and 14 (33%) passed our quality control standards (indicating that they were expressed in at least 85% of all SAMARA subjects) and were included in the final processed dataset used for the GSEA (Table 3.2).

The primary GSEA was completed in the entire cohort of 143 subjects; due to the known confounding effects of race and gender on genes expression,[132, 133] secondary analyses were conducted using the subset of male subjects (n=75), Caucasian subjects (n=111), and Caucasian male subjects (n=64). The number of permutations for each analysis was set at 1000, with a p-value of <0.05 considered significantly enriched.

Results

Study Population

We enrolled 143 subjects undergoing diagnostic cardiac catheterization; the study population characteristics are shown in Table 3.3. Subjects were on average 74±6 years of age and a total of 93 subjects (65%) had obstructive CAD while 50 (35%) did not have obstructive CAD. The majority of subjects were Caucasian (78%), male (52%) and had multiple comorbidities including hyperlipidemia (71%) and hypertension (88%). Use of medications for the treatment of CAD was common including statins (68%), aspirin (78%) and clopidogrel (27%). Population characteristics were similar in subjects diagnosed with obstructive CAD and subjects without an obstructive CAD diagnosis (Table 3.3). However, individuals with obstructive CAD were more likely to be male (61%) compared to individuals without obstructive CAD (36%). Consistent with current clinical practice guidelines, individuals with obstructive CAD were also more likely to be receiving medications used in the treatment of CAD, including aspirin, clopidogrel, and statins (Table 3.3).

Arachidonic Acid Metabolism Pathway Expression in Obstructive CAD

The results of the arachidonic acid metabolism pathway GSEA are summarized in Table 3.4. The arachidonic acid metabolism pathway was not significantly enriched in subjects with obstructive CAD (p=0.526), indicating that relative to global gene expression changes, the arachidonic acid metabolism pathway as a whole was not dysregulated in individuals

with obstructive CAD compared to individuals without obstructive CAD. Similar to the analysis in the entire cohort, significant enrichment of the arachidonic acid pathway was not observed after stratifying the cohort and limiting the analysis to Caucasians (p=0.255), males (p=0.288), or Caucasian male subjects (p=0.425, Table 3.4).

Table 3.5 lists each gene in the arachidonic acid metabolism pathway in rank order of its correlation with obstructive CAD. The overall rank of the correlation of each gene relative to the 10,096 unique annotated genes in the correlation analysis, the direction of the association with CAD (positive indicates higher expression in individuals diagnosed with obstructive CAD) and whether or not the gene was part of the core enrichment subset are displayed. Although the overall GSEA was not statistically significant, the core enrichment subset for this analysis shows that the enrichment signal was strongest for *EPHX2*, *PTGS1*, and *PLA2G12A*, which were negatively correlated with the presence of obstructive CAD (Table 3.5).

Since *EPHX2* was found to demonstrate one of the strongest inverse correlations relative to all genes expressed in PBMCs (rank 9,975 out of 10,096), we conducted an exploratory analysis using ANOVA to determine whether this individual gene in the arachidonic acid pathway was differentially expressed in subjects with obstructive CAD. We found that *EPHX2* was significantly suppressed by 18±4% (p=0.007) in individuals with obstructive compared to individuals without obstructive CAD (Figure 2.1).

Discussion

Preclinical and epidemiologic studies have demonstrated that arachidonic acid metabolism is important in the regulation of vascular inflammation, and the subsequent development and progression of CAD. In this study, we utilized GSEA to determine whether arachidonic acid metabolism pathway gene expression is “enriched” in PBMCs of older individuals with obstructive CAD. Our analysis did not demonstrate significant enrichment of

arachidonic acid metabolism pathway gene expression in individuals with obstructive CAD. Similarly, GSEA of population subsets including Caucasians, males, and Caucasian male subjects did not demonstrate significant enrichment in individuals with obstructive CAD, suggesting that the coordinated dysregulation of key genes involved in the metabolism of arachidonic acid in PBMCs is not associated with the development of obstructive CAD in older humans.

It is well-established that 5-lipoxygenase (5-LO) pathway derived leukotrienes are pro-inflammatory chemoattractant molecules that activate leukocytes and contribute to recruitment of monocytes to the vessel wall.[51] Conversely, COX pathway enzymes metabolize arachidonic acid to prostaglandins which may have pro- or anti-inflammatory effects.[42, 134] While COX-derived prostaglandins generally increase vascular permeability and leukocyte infiltration, their actions are tissue specific and inhibition of prostaglandin biosynthesis has yielded conflicting effects on cardiovascular inflammatory responses and atherosclerosis in pre-clinical models.[134] Similar to the COX pathway, CYP-mediated arachidonic acid metabolism drives both pro- and anti-inflammatory processes in the vasculature. CYP ω-hydroxylase derived 20-hydroxyeicosatetraenoic acid (HETE) elicits pro-inflammatory vasoconstrictive effects, while CYP epoxygenase derived epoxyeicosatrienoic acids (EETs) are anti-inflammatory and cardioprotective.[74, 86] Therefore, although our results demonstrated that arachidonic acid metabolism pathway gene expression was not dysregulated as a whole in subjects with obstructive CAD, we cannot dismiss the possibility that the individual (i.e. COX, LOX, and CYP) pathways of arachidonic acid metabolism may be important mediators of systemic and vascular inflammation, and the subsequent development and progression of CAD.

Our analysis has limitations that must be noted. First, our cohort consisted only of individuals ≥65 years of age. While our findings cannot be extrapolated to other population subsets, older individuals are at high-risk for CAD and the pathophysiology of the

development and progression of CAD has previously remained largely unexplored in this population subset. Second, PBMC RNA expression is a surrogate for capturing biological processes taking place at the site of the atherosclerotic lesion, and the CYP, COX, and LOX pathways are metabolically active in other cell types that also are important in CAD, including endothelial cells, platelets, cardiomyocytes, and hepatocytes.[34] Furthermore, multiple arachidonic acid metabolism pathway genes either were not on the microarray or did not pass our quality control analysis and therefore were not included in the GSEA. Moreover, some proteins including CYPs are regulated at the post-transcriptional level.[135] Therefore, it is possible that individual components of the arachidonic acid metabolism pathway that are important in the pathophysiology of CAD may not have been captured in our pathway analysis of genes that metabolize arachidonic acid to biologically active eicosanoids in PBMCs. Furthermore, previous genetic association studies have demonstrated that individual pathways of arachidonic acid metabolism (i.e. COX, LOX, and CYP) are important in the development of CAD. Lastly, although our analysis is one of the largest microarray studies conducted in patients at high-risk for CAD, some of the subgroup comparisons contained small numbers of subjects and therefore had limited power to detect enrichment of the arachidonic acid metabolism pathway. The absence of a significant association at the RNA level between arachidonic acid metabolism pathway gene expression in PBMCs and obstructive CAD, suggests that the parallel components of the overall arachidonic acid metabolism pathway are not coordinately dysregulated in the presence of obstructive CAD in older individuals. However, the limitations described above highlight the need for candidate pathway approaches to rigorously evaluate the functional relationship between individual pathways of arachidonic acid metabolism and the pathogenesis and progression of CAD.

Accumulating preclinical and epidemiologic evidence suggests that CYP-mediated eicosanoid metabolism is important in the regulation of vascular inflammation. The CYP-

epoxygenase pathway exerts potent anti-inflammatory effects while the CYP ω-hydroxylase pathway exerts pro-inflammatory effects.[74, 86] In our study, only two genes from the CYP pathway were found two be expressed on PBMCs, the CYP ω-hydroxylase *CYP4F2* and the CYP-epoxygenase pathway gene *EPHX2* which encodes soluble epoxide hydrolase (sEH), the enzyme responsible for metabolizing EETs to the less biologically active dihydroxyeicosatrienoic acids (DHETs). While our analysis of gene expression in PBMCs did not demonstrate significant dysregulation of the arachidonic acid metabolism pathway as a whole, our exploratory analysis found that *EPHX2* was suppressed in subjects with obstructive CAD. It is important to note that the association between *EPHX2* expression and CAD score did not exceed the *a priori* absolute correlation coefficient of ≥0.25 in our global analysis of gene expression (r_s=-0.20, p=0.020) and therefore was not included in our list of 512 differentially expressed genes (Chapter II). In addition, this exploratory finding requires validation by quantitative real time-polymerase chain reaction (qRT-PCR) and replication in an independent cohort; however, suppression of *EPHX2* in the presence of CAD is consistent with our previous report that sEH metabolic function is suppressed in stable CAD patients under 65 years of age compared to healthy volunteers.[136] Moreover, previous studies have demonstrated that myocardial *EPHX2* expression is suppressed in individuals with ischemic heart failure compared to control subjects.[137] These findings suggest that the presence of CVD may lead to a compensatory suppression of sEH-mediated EET hydrolysis in humans that was not captured by our global analysis of arachidonic acid pathway gene expression in PBMCs. Given the emerging importance of CYP-mediated eicosanoid metabolism in the regulation of pathological processes integral to CAD in preclinical models,[74] future studies evaluating the association between inter-individual variation in CYP-derived eicosanoids at the metabolite level and the pathogenesis and progression of CAD in humans are warranted.

Conclusions

In summary, our analysis in older individuals presenting for coronary angiography did not demonstrate that arachidonic acid metabolism pathway gene expression is coordinately dysregulated in PBMCs of individuals with obstructive CAD. However, future studies are warranted to define the functional link between candidate pathways of arachidonic acid metabolism and the pathogenesis and progression of CAD.

Tables

Table 3.1. Genes in the arachidonic acid metabolism pathway from the KEGG database.

PLA	CYP	COX	LOX
PLA2G10	*CYP2B6*	*PTGDS*	*ALOX12*
PLA2G12A	*CYP2C18*	*PTGES*	*ALOX12B*
PLA2G12B	*CYP2C19*	*PTGES2*	*ALOX15*
PLA2G1B	*CYP2C8*	*PTGIS*	*ALOX15B*
PLA2G2A	*CYP2C9*	*PTGS1*	*ALOX5*
*PLA2G2C**	*CYP2E1*	*PTGS2*	*ALOX5AP*
PLA2G2D	*CYP2J2*	*TBXAS*	*LTA4H*
PLA2G2E	*CYP2U1*		*LTC4S*
PLA2G2F	*CYP4A11*		
PLA2G3	*CYP4A22**		
PLA2G4A	*CYP4F2*		
PLA2G4B	*CYP4F3*		
*PLA2G4E**	*EPHX2*		
PLA2G5			
PLA2G6			

*Included in the arachidonic acid metabolism pathway from the KEGG database but not represented on the microarray.

Table 3.2. Arachidonic acid pathway genes in final analysis.

PLA	CYP	COX	LOX
PLAG4A	*CYP4F2*	*PTGS1*	*ALOX5*
PLA2G12A	*EPHX2*	*PTGS2*	*ALOX5AP*
PLA2G4B		*PTGES2*	*LTA4H*
PLA2G6		*PTGDS*	
		TBXAS1	

Table 3.3. Demographics are presented for all subjects, and according to obstructive CAD status.

Characteristic	All	Obstructive CAD	No Obstructive CAD
N	143	93	50
Age (years)	73.9±6.3	74±6.3	73±6.2
Male gender (%)	75 (52%)	57 (61%)	18 (36%)*
Caucasian (%)	111 (78%)	74 (80%)	37 (74%)
Body mass index (kg/m^2)	29±6.6	29±6.3	30±7.1
Current smoker (%)	7 (5%)	4 (4%)	3 (6%)
Diabetes (%)	55 (38%)	38 (41%)	17 (34%)
Hyperlipidemia (%)	102 (71%)	74 (80%)	28 (56%)*
Hypertension (%)	126 (88%)	82 (88%)	44 (88%)
Previous myocardial infarction	44 (31%)	41 (44%)	3 (6%)*
Multivessel disease	62 (43%)	62 (67%)	0 (0%)*
Systolic blood pressure (mmHg)	142±20	143±20	141±20
Diastolic blood pressure (mmHg)	78±13	77±13	78±12
Statin use (%)	97 (68%)	74 (80%)	23 (46%)*
Aspirin use (%)	111 (78%)	78 (84%)	33 (66%)*
Clopidogrel use (%)	39 (27%)	33 (36%)	6 (12%)*

Data presented as mean ± standard deviation or count (proportion).

*P<0.05 vs. individuals with obstructive CAD.

Age, body mass index, and diastolic blood pressure were not normally distributed and were log-transformed prior to analysis.

Table 3.4. Results of Arachidonic Acid Metabolism Pathway Gene Set Enrichment Analysis.

Population	Obstructive CAD (yes vs. no)
All (n=143)	0.526
Caucasians only (n=75)	0.255
Males only (n=111)	0.288
Caucasian Males only (n=64)	0.425

P-values are presented for each of the Gene Set Enrichment Analyses.

Table 3.5. Summary of GSEA results for the arachidonic acid metabolism pathway in patients with obstructive CAD.

Rank	Gene	Overall Rank*	Direction	Core Enrichment^
1	*PLA2G4A*	827	Positive	No
2	*CYP4F2*	1950	Positive	No
3	*LTA4H*	2540	Positive	No
4	*ALOX5*	2703	Positive	No
5	*ALOX5AP*	2830	Positive	No
6	*PTGDS*	3463	Positive	No
7	*TBXAS1*	3882	Positive	No
8	*PLA2G6*	4615	Positive	No
9	*PTGS2*	5062	Negative	No
10	*PTGES2*	6079	Negative	No
11	*PLA2G4B*	6577	Negative	No
12	*PLA2G12A*	8965	Negative	Yes
13	*PTGS1*	9216	Negative	Yes
14	*EPHX2*	9975	Negative	Yes

*Overall rank of 10,096 unique annotated genes in correlation analysis with obstructive CAD. The direction of the association indicates whether the gene was expressed at higher (positive) or lower (negative) levels in subjects with obstructive CAD.

^The Core Enrichment subset of genes is the core of a gene set that accounts for the observed signal. Since the overall GSEA was not statistically significant (p=0.526), the core enrichment subset for this analysis indicates that the enrichment signal was strongest in *EPHX2*, *PTGS1*, and *PLA2G12A*, which were inversely correlated with the presence of obstructive CAD.

Figures

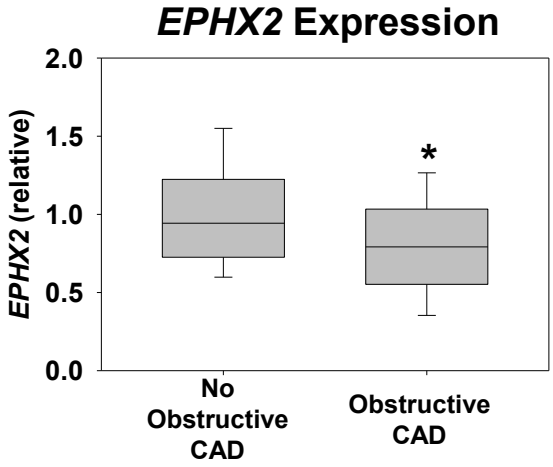

Figure 3.1. *EPHX2* expression in PBMCs according to obstructive CAD status.

The expression of *EPHX2* in PBMCs is displayed relative to individuals without obstructive CAD. The horizontal line within the box represents the median, the edges of the box represent the 25th and 75th percentiles, and the error bars represent the 10th and 90th percentiles. *EPHX2* was found to be significantly lower in individuals with obstructive CAD (n=93) compared to individuals without obstructive CAD (n=50). *P=0.007 vs. subjects without obstructive CAD.

CHAPTER IV

CYTOCHROME P450-DERIVED EICOSANOIDS AND VASCULAR DYSFUNCTION IN CORONARY ARTERY DISEASE PATIENTS[1]

Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide and despite recent advances, novel therapies are needed to further improve outcomes. Inflammation and impaired arterial vasodilation (endothelial dysfunction) are key drivers of the pathogenesis and progression of CAD in preclinical models and humans.[9] It is well established that elevated circulating biomarkers of systemic (C-reactive protein)[24] and vascular (chemokines, cellular adhesion molecules [CAMs])[12, 13] inflammation and impaired physiologic measures of endothelial function (brachial artery flow-mediated dilation [FMD])[22] are predictive of poor prognosis in patients with established CAD. Therefore, CAD patients with persistent vascular inflammation and/or endothelial dysfunction, despite treatment with current standards of care, may be candidates for adjunct therapeutic strategies specifically designed to improve vascular function. Identification of the key pathways that regulate these phenotypes in humans, however, is essential to facilitate the development of targeted therapies for these high-risk subsets of the population.

Accumulating preclinical and epidemiologic evidence has emerged to suggest that modulation of cytochrome P450 (CYP)-mediated eicosanoid metabolism may be a viable

[1] Schuck RN, Theken KN, Edin ML, Caughey M, Bass A, Ellis K, Tran B, Steele S, Simmons BP, Lih FB, Tomer KB, Wu MC, Hinderliter AL, Zeldin DC, Stouffer GA, and Lee CR. Atherosclerosis, 2013;227(2):442-448.

clinical therapeutic strategy for the management of cardiovascular disease.[74, 86] The CYP2J and CYP2C epoxygenases metabolize arachidonic acid to four epoxyeicosatrienoic acid (EET) regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET), which possess potent vasodilatory and anti-inflammatory effects. The EETs, however, are rapidly metabolized to less active dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). In parallel, CYP4F and CYP4A ω-hydroxylases generate 20-hydroxyeicosatetraenoic acid (20-HETE), which exhibits potent vasoconstrictive and pro-inflammatory effects. Inhibition of sEH mediated EET hydrolysis and CYP ω-hydroxylase mediated 20-HETE biosynthesis each elicit potent protective effects in preclinical models of endothelial dysfunction and hypertension, nuclear factor (NF)-κB dependent vascular inflammation, atherosclerosis, and myocardial ischemia-reperfusion injury.[73-75, 77, 78, 88, 138] In humans, genetic epidemiology studies have shown that functional polymorphisms in CYP epoxygenases (*CYP2J2* and *CYP2C8*), sEH (*EPHX2*), and CYP ω-hydroxylases (*CYP4F2* and *CYP4A11*) are associated with the development of cardiovascular disease.[81, 83, 92]

Furthermore, we recently reported that patients with established atherosclerotic cardiovascular disease exhibit higher plasma EET levels and higher 14,15-EET:DHET ratios compared to healthy individuals at low risk for cardiovascular disease.[136] Conversely, no significant differences in 20-HETE levels were observed. Due to the vascular protective effects of EETs in preclinical models, these data suggest that the presence of cardiovascular disease may lead to a compensatory suppression of sEH-mediated EET hydrolysis in humans. Importantly, pharmacologic inhibitors of sEH[139] and CYP ω-hydroxylase metabolism[140] are currently in development. Although these novel therapies are hypothesized to improve prognosis in CAD patients by eliciting protective effects in the vasculature via increasing EET and decreasing 20-HETE levels, respectively, it remains unknown whether CAD patients with dysregulated CYP-mediated eicosanoid metabolism exhibit more advanced vascular dysfunction. A more thorough understanding of the role of

CYP-derived EETs and 20-HETE in the regulation of vascular function in patients with established CAD offers enormous potential to identify subsets of the population with dysregulated eicosanoid metabolism (i.e., low EET and/or high 20-HETE levels) who may be most likely to derive benefit from these novel therapies in development.

Therefore, the primary objective of this study was to characterize the relationship between inter-individual variation in CYP-mediated eicosanoid metabolism and key vascular function phenotypes in patients with stable atherosclerotic cardiovascular disease, and determine whether the subset of individuals with enhanced sEH and CYP ω-hydroxylase metabolic function exhibit endothelial dysfunction and advanced inflammation.

Methods

Study Population

A cohort of 106 individuals with established and stable CAD, defined as ≥50% stenosis in one or more major epicardial coronary arteries by coronary angiography, were identified in the University of North Carolina (UNC) Cardiac Catheterization Laboratory between October 2007 and November 2010.[136, 141] Exclusion criteria included pregnancy, atrial fibrillation, left-ventricular systolic dysfunction (ejection fraction ≤35%), current use of long-acting nitrates or insulin, active autoimmune disease, history of severe aortic stenosis, history of solid organ transplant or dialysis, or history of cancer within the previous 5 years. Eligible participants provided written informed consent and returned to the UNC Clinical and Translational Research Center 65±35 days after their index catheterization for a single morning study visit and blood sample collection after fasting overnight and withholding their morning medications. All study visits took place at least 7 days following the index catheterization, and all participants were clinically stable and chest pain free at the time of their study visit.

Participants were instructed to refrain from tobacco products, caffeine, and vigorous exercise the morning of the study visit, and from use of vitamin C, vitamin E, fish oil, niacin or arginine supplements, oral decongestants, non-steroidal anti-inflammatory drugs (other than low-dose aspirin), or phosphodiesterase-5 inhibitors for at least seven days prior to the study visit. Individuals who experienced a respiratory tract infection within four weeks of the study visit were not eligible to participate, but could be scheduled at a later date. The study protocol was approved by the UNC Biomedical Institutional Review Board and conducted in accordance with institutional guidelines.

Evaluation of Endothelial Function

Endothelial dysfunction is a physiologic manifestation of vascular inflammation secondary to impaired nitric oxide availability and function that is associated with future cardiovascular events in multiple patient populations.[142]

Endothelium-dependent vasodilation was assessed by brachial artery FMD using a 12.5 MHz linear-array transducer (Philips HDI 5000 system) as previously described.[141, 143] All recordings were taken in the morning in a dimly lit room by the same sonographer. Following 10 minutes of rest, baseline brachial artery diameter was measured at end diastole from the lumen-intimal interface of the proximal and distal walls and calculated by averaging 10 consecutive frames. Reactive hyperemia was induced by inflating a blood pressure cuff around the right forearm for five minutes to a pressure of at least 70 mmHg greater than the systolic blood pressure. Upon cuff release, recordings were taken for 90 seconds and peak brachial artery diameter was assessed by averaging three consecutive frames. FMD was calculated as the peak percent change in brachial artery diameter from baseline [= $100*(\text{diameter}_{\text{Peak}} - \text{diameter}_{\text{Baseline}}) / (\text{diameter}_{\text{Baseline}})$]. Following 10 minutes of rest, endothelium-independent vasodilation was quantified as the percent change in arterial

diameter five minutes after sublingual administration of a 0.4 mg nitroglycerin tablet. All data were analyzed by Brachial Tools software (Medical Imaging Applications, Coralville, IA).

Quantification of Inflammatory Biomarkers

It is well-established that circulating biomarkers of inflammation are associated with prognosis in patients with established CAD.[9] Individuals with elevated high sensitivity C-reactive protein (hs-CRP, a systemic inflammatory mediator produced in the liver that correlates with cytokines including interleukin-6),[144] CAMs (inflammatory mediators expressed on endothelial cells that mediate leukocyte and platelet adhesion) and monocyte chemoattractant protein-1 (MCP-1, a chemokine synthesized in monocytes and endothelial cells that drives monocyte recruitment to the vascular wall) levels, and genetic predisposition to higher epithelial neutrophil-activating protein (ENA)-78 levels (a chemokine synthesized in neutrophils and endothelial cells that drives neutrophil recruitment to the vascular wall) have each been associated with poorer survival in patients with established CAD.[12, 13, 17]

Venous blood was collected from each study participant, and plasma and serum were separated by centrifugation. In fresh serum, hs-CRP was quantified by latex-enhanced turbidimetric immunoassay using the VITROS® 5600 Chemistry System (Ortho-Clinical Diagnostics, Inc., Rochester, NY) by UNC McLendon Clinical Laboratories. The remaining serum and plasma were aliquoted and stored at -80°C pending analysis. Plasma concentrations of CAMs (E-selectin and P-selectin) and the neutrophil (ENA-78) and monocyte (MCP-1) chemokines were quantified using the Human Adhesion Molecule and Human Cytokine Fluorokine® Multi-Analyte Profiling Kits (R&D Systems, Minneapolis, MN), respectively, with fluorescence detection on the Bio-Plex 200 System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Quantification of Plasma Eicosanoids

Plasma eicosanoids were quantified after solid phase extraction by high-performance liquid chromatography followed by tandem mass spectrometry (HPLC-MS/MS) as previously described.[81, 136, 145] HyperSep Retain PEP SPE cartridges (Thermo Scientific, Rockford, IL) were pre-conditioned with a solution of 0.1% acetic acid/5% methanol and spiked with 30 ng each of 10,11-epoxyheptadecanoic acid and 10,11-dihydroxynonadecanoic acid (internal standards). Plasma (0.25 mL) was diluted in 0.1% acetic acid/5% methanol containing 0.009 mmol/L butylated hydroxytoluene and added to the column. Samples were then washed with two volumes of 0.1% acetic acid/5% methanol, eluted in 1 mL of acetonitrile, dried under nitrogen gas at 37°C, and reconstituted in 40% ethanol.

Arachidonic acid derived metabolites from the CYP epoxygenase pathway (8,9- EET, 11,12-EET, 14,15-EET, 5,6- DHET, 8,9-DHET, 11,12-DHET, and 14,15-DHET) and CYP ω-hydroxylase pathway (20-HETE), and linoleic acid derived metabolites from the CYP epoxygenase pathway (12,13-epoxyoctadecenoic acid [EpOME] and 12,13-dihydroxyoctadecenoic acid [DHOME]) were separated by reverse phase HPLC on a 1x150 mm, 5μm Luna C18(2) column (Phenomenex, Torrance, CA) and quantified using a MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with negative mode electrospray ionization and multiple reaction monitoring. Data were captured and analyzed using Analyst 1.5.1 software. Relative response ratios of each analyte were used to calculate concentrations and extraction efficiency for each sample was calculated based on recovery of the internal standards. For samples in which the concentration fell below the lowest standard, a value of one half the lowest standard was imputed. Analytes for which more than 20% of the values were imputed were dropped from the statistical analysis.[146] Consistent with prior analyses,[147] plasma 11,12-EET concentrations were below the lower limit of detection in 68 of 106 samples (64%), and therefore excluded from the analysis.

Statistical Analysis

Data are presented as mean ± standard deviation or median (interquartile range) unless otherwise indicated. CYP-derived eicosanoids and circulating biomarkers of inflammation did not follow the normal distribution and therefore were log-transformed prior to analysis. In order to account for batch variation in the quantification of inflammatory biomarkers, data within each batch were standardized using a z-score [i.e. (subject value – batch mean)/batch standard deviation], as described.[105] Correlations among each vascular function phenotype were determined by Pearson's correlation. A significant correlation was observed between E-selectin and P-selectin ($r=0.599$, $P<0.001$). In order to minimize redundancy in our analysis, a consolidated 'CAM score' phenotype was calculated in each individual, as described,[105] by summing the z-scores of E-selectin and P-selectin. No significant correlations were observed among the final five biomarkers of vascular function (Table 4.1), indicating these biomarkers represent five distinct phenotypes.

In order to characterize the association between inter-individual variation in CYP-mediated eicosanoid metabolism and key vascular function phenotypes, plasma 20-HETE levels (the bioactive metabolite of the CYP ω-hydroxylase enzymes) were utilized as a biomarker of CYP ω-hydroxylase pathway function. The plasma 14,15-EET:DHET ratio (a sensitive *in vivo* biomarker of sEH metabolic function)[73, 81] and the sum of EET levels in plasma (the bioactive metabolites of CYP epoxygenase enzymes) were utilized as biomarkers of CYP epoxygenase pathway function as previously described (Figure 4.1).[136]

For the primary analysis, associations between circulating biomarkers of CYP-mediated eicosanoid metabolism (20-HETE, 14,15-EET:DHET ratio, and sum EETs) and five distinct phenotypic indices of vascular function (FMD, CAM score, MCP-1, ENA-78, and hs-CRP) were evaluated by Pearson's correlation. A secondary analysis was conducted using a model that adjusted for potential demographic (age, race, gender) and clinical (smoking

status, diabetes, obesity, multivessel disease, hypertension, renin-angiotensin system inhibitor use, time post-catheterization [7-30 days, 31-90 days, >90 days]) confounders that associated with the biomarkers of CYP eicosanoid metabolism and/or phenotypic indices of vascular function in our population.[136] In order to account for the potential confounding effects of medications that may impact vascular function and/or CYP-mediated eicosanoid metabolism, each analysis was repeated after adding aspirin, clopidogrel, beta-blocker, or statin use to the adjusted model. We also conducted stratified analyses limited to subjects receiving aspirin (n=103), clopidogrel (n=83), beta-blockers (n=89), or statins (n=83).

In order to further assess the eicosanoid-vascular function relationships, vascular phenotypes were compared across eicosanoid metabolism tertiles by analysis of variance (ANOVA) and a post-hoc Student Newman-Keuls test. To minimize the impact of the multiple statistical tests required for this analysis, a false discovery rate (FDR) q-value was calculated for each comparison in our primary analysis, which is defined as the expected proportion of statistical tests deemed significant that are actually false-positives.[148] Only q-values for statistically significant findings ($p<0.05$) are presented. A post-hoc power analysis was completed to assess the statistical power of our study. All statistical analyses were performed using SAS Version 9.2 (SAS Institute, Cary, NC).

Results

Study Population

The population characteristics are shown in Table 4.2. The majority of patients had advanced CAD, with 66% exhibiting multivessel disease at their index catheterization, and comorbidities including hypertension (81%), obesity (54%), and diabetes (24%). Medication utilization and revascularization rates were consistent with current clinical practice guidelines.

CYP ω-hydroxylase Pathway

The relationships between inter-individual variation in CYP-derived eicosanoid levels and vascular function are provided in Table 4.3. Plasma 20-HETE levels were significantly associated with endothelial dysfunction, such that patients with higher levels of 20-HETE had lower FMD (Figure 4.2A, r=-0.255, p=0.010, q=0.048). A significant inverse association was also observed in the adjusted model (r=-0.223, p=0.033). Similarly, a stepwise trend of lower FMD was observed across increasing tertiles of 20-HETE (Figure 4.2B, p for trend=0.080); however, statistically significant differences were not observed between each tertile. Circulating 20-HETE levels were not associated with either nitroglycerin-mediated dilation (r=-0.068, p=0.495) or baseline brachial artery diameter (r=0.074, p=0.455), indicating that the mechanism underlying the association between 20-HETE and FMD is endothelium-dependent and is not explained by differences in arterial size.

A positive relationship between 20-HETE levels and the CAM score was also observed (unadjusted: r=0.191, p=0.051; adjusted model: r=0.222, p=0.031); however, the association was not statistically significant in the primary analysis. In contrast, no association was observed between 20-HETE and either hs-CRP or chemokine levels (Table 4.3). Similar relationships between 20-HETE levels and each vascular function phenotype were observed after adjusting for or stratifying by aspirin, clopidogrel, beta blocker, or statin use (data not shown).

CYP Epoxygenase Pathway

In contrast to the observed relationship between 20-HETE and endothelial function, no association was observed between FMD and either 14,15-EET:DHET ratios or sum EETs (Table 4.3). Lower 14,15-EET:DHET ratios (indicative of higher sEH metabolic function) were, however, significantly associated with higher circulating levels of MCP-1 (Figure 4.3A,

r=-0.252, p=0.009, q=0.048). This association was also significant in the adjusted model (r=-0.260, p=0.011), and across tertiles (Figure 4.3B). A significant inverse correlation was also observed between MCP-1 levels and both the sum EET:DHET ratio (r=-0.245, p=0.012) and 12,13-epoxyoctadecenoic acid (EpOME):dihydroxyoctadecenoic acid (DHOME) ratio (r=-0.201, p=0.040). In addition, lower 14,15-EET:DHET ratios were associated with higher CAM scores (Figure 4.3C, r=-0.216, p=0.027, q=0.101), such that those in the lowest tertile had a significantly higher CAM score compared to tertiles 2 and 3 (Figure 4.3D). The inverse relationship between CAM score and 14,15-EET:DHET ratio, however, was not statistically significant in the adjusted model (r=-0.190, p=0.065). Similar results were observed with the sum EET:DHET ratio and 12,13-EpOME:DHOME ratio (data not shown).

Consistent with the relationship observed with 14,15-EET:DHET ratios, lower sum EET levels were associated with higher MCP-1 levels (Figure 4.4A, r=-0.283, p=0.003, q=0.045). A significant inverse association was also observed in the adjusted model (r=-0.243, p=0.017). Furthermore, those with sum EET levels in the lowest two tertiles exhibited significantly higher MCP-1 levels compared to those in the highest tertile (Figure 4.4B). When analyzed separately, a significant inverse relationship was observed between MCP-1 and both 8,9-EET (r=-0.244, p=0.012) and 14,15-EET (r=-0.284, p=0.003) levels. No association was observed between sum EETs or 14,15-EET:DHET ratio and either hs-CRP or ENA-78 levels (Table 4.3). Similar relationships between both 14,15-EET:DHET ratio and sum EETs and each vascular function phenotype were observed after adjusting for or stratifying by aspirin, clopidogrel, beta-blocker, or statin use (data not shown).

Discussion

Preclinical and genetic epidemiologic studies suggest that increasing CYP-derived EETs and/or decreasing 20-HETE levels may have utility as a vascular protective therapeutic strategy in patients with cardiovascular disease. The functional relationship between CYP-

mediated eicosanoid metabolism and vascular dysfunction in humans with established cardiovascular disease, however, has not been studied to date. This study demonstrated that higher 20-HETE levels were associated with lower brachial artery FMD and higher CAM levels and lower 14,15-EET:DHET ratios were associated with elevated MCP-1 and CAM levels in a population of patients with stable CAD. These findings are consistent with the vascular effects of 20-HETE and EETs in preclinical models, and suggest that enhanced CYP ω-hydroxylase and sEH metabolic function predispose stable CAD patients to more advanced endothelial dysfunction and vascular inflammation, respectively.

It is well-established that biomarkers of vascular function are associated with prognosis in patients with established CAD.[9] For example, persistently elevated circulating levels of hs-CRP, CAMs and MCP-1, or genetic predisposition to higher ENA-78 levels, have each been associated with poorer survival in patients with established CAD.[12, 13, 17, 24] Similarly, CAD patients with persistently impaired FMD despite optimized therapy have a higher risk of cardiovascular events, suggesting that adjunct therapies that improve FMD may subsequently improve prognosis.[22] Identification of key regulators of these distinct vascular phenotypes predictive of prognosis offers enormous potential to facilitate the development of targeted therapies designed to improve vascular function and prognosis in high-risk subsets of the population.

The observed inverse association between circulating 20-HETE levels and brachial artery FMD is consistent with preclinical studies in which 20-HETE directly induces reactive oxygen species (ROS) production and endothelial nitric oxide synthase uncoupling, reduces NO availability, and impairs endothelial-dependent vasodilation.[88] An inverse association between urinary excretion of 20-HETE, which is reflective of renal 20-HETE biosynthesis,[138] and FMD has previously been reported in humans without known CAD.[94] Our findings with circulating 20-HETE are consistent with this report; however, we are the first to demonstrate an association between 20-HETE and FMD in humans with established CAD. We also

observed a positive relationship between plasma 20-HETE levels and circulating CAMs that is consistent with preclinical studies demonstrating that 20-HETE directly induces CAM expression in endothelial cells through activation of NF-κB.[87] In our previous case-control analysis, we did not observe a significant difference in 20-HETE levels in stable CAD patients compared to healthy individuals; however, CAD patients who were not treated with a renin-angiotensin system inhibitor exhibited modestly higher 20-HETE levels compared to healthy individuals.[136] Our current findings suggest that despite being treated with current standard of care therapies (including renin-angiotensin system inhibitors), CAD patients with the highest 20-HETE levels may be predisposed to more advanced endothelial dysfunction and vascular inflammation, and therapeutic strategies that directly decrease 20-HETE biosynthesis may improve vascular function in this high-risk population.

Consistent with a series of preclinical studies and our hypothesis, we are also the first to report that lower EET:DHET ratios, a biomarker of enhanced sEH metabolic function, were associated with higher plasma MCP-1 and CAM levels. Indeed, EETs exert potent anti-inflammatory effects in the vasculature in preclinical models via attenuation of NF-κB activation and chemokine and CAM expression,[75, 77] and these effects are potentiated via inhibition of sEH.[74] These data are consistent with a recent report demonstrating that CYP2J2 and CYP2C8 are expressed in human monocytes, and CYP epoxygenase metabolic function attenuates monocyte/macrophage activation.[149] In addition, a recent study in mice demonstrated that sEH activity in bone marrow-derived cells, the primary site of chemokine biosynthesis, is a key regulator of plasma epoxide:diol ratios.[150] We previously reported that plasma epoxide:diol ratios were significantly higher in patients with established CAD compared to healthy individuals at low risk for cardiovascular disease.[136] Due to the aforementioned vascular protective effects of EETs, these data suggest that the presence of cardiovascular disease may lead to a compensatory suppression of sEH metabolic function and higher EET levels in humans. However, our current findings suggest that, in spite of a

potential compensatory increase in EET levels overall, the subset of CAD patients with the highest sEH metabolic function may be predisposed to more advanced vascular inflammation. Consequently, therapeutic strategies that further increase EET levels, most notably sEH inhibitors, may represent an effective secondary prevention strategy by attenuating vascular inflammation and improving prognosis in this high-risk population.

In contrast, we did not observe an association between 14,15-EET:DHET ratio and FMD which is surprising given the known vasodilatory properties of EETs in preclinical models and our previous finding that *EPHX2* genotype is associated with vasodilator responses in healthy individuals.[82] In addition to the different populations, it should be noted that brachial artery FMD (an index of NO bioavailability and conduit arterial function) was evaluated in the current study while bradkyinin-induced changes in forearm blood flow (a phenotype more reflective of microvascular function) was evaluated in the previous study. Although these contrasting results may not be surprising given the more prominent role of EETs in the regulation of microvascular compared to macrovascular tone in preclinical models,[86] it is important to note that our current findings are consistent with previous studies demonstrating that CYP epoxygenase inhibition does not impair endothelium-dependent vasodilator responses in CAD patients *in vivo,*[151] or in isolated coronary arterioles from patients undergoing cardiac surgery, [152] due to the presence of enhanced oxidative stress. Collectively, these data suggest that the vasodilatory effects of CYP-derived EETs may be overwhelmed and masked by the presence of ROS in populations with underlying vascular inflammation and remodeling, such as atherosclerotic cardiovascular disease. This hypothesis requires rigorous investigation in prospective interventional studies.

No associations were observed between biomarkers of CYP-mediated eicosanoid metabolism and hs-CRP. While chemokines and CAMs mediate inflammation in the vasculature, hs-CRP is an acute-phase reactant released from the liver indicative of systemic inflammation.[153] Consequently, our findings indicate that CYP-derived eicosanoids

may be important in the regulation of vascular, but not hepatic or systemic, inflammation in humans. This is consistent with previous observations in preclinical models demonstrating that the hepatic inflammatory response to endotoxin is not attenuated in *Ephx2* knockout mice.[154] Furthermore, no relationship between CYP-mediated eicosanoid metabolism and the neutrophil chemokine ENA-78 was observed. We have reported that genetic potentiation of the CYP epoxygenase pathway attenuates induction of ENA-78 expression and neutrophil infiltration in mice.[77] In contrast, CYP epoxygenase enzymes are not expressed in human polymorphonuclear cells,[149] suggesting the presence of species differences in the contribution of CYP-derived eicosanoids to the regulation of neutrophil activation.

Our analysis has limitations that must be acknowledged. First, although the observed associations were consistent with prior preclinical studies, the cross-sectional design does not allow us to establish a cause-and-effect relationship between eicosanoid metabolism and vascular function. Second, our analysis included multiple statistical comparisons and was limited by its relatively small sample size. To account for the possibility of false-positive findings, we calculated a FDR for each comparison. All statistically significant associations had a FDR of 10% or less, and were in directions consistent with prior preclinical evidence, giving us a higher level of confidence in our results. Furthermore, at α=0.05, there was greater than 80% power to detect an r^2 of 0.07 for each eicosanoid metabolism-vascular phenotype relationship, which is similar in magnitude to the well-established and clinically relevant association observed between body mass index and hs-CRP in previous studies.[153] This demonstrates that we had ample statistical power to detect biologically meaningful and clinically relevant relationships in the current study. Importantly, validation of the observed relationships in an independent cohort will ultimately be necessary. Lastly, the population under investigation had established atherosclerotic cardiovascular disease and thus was being treated according to current clinical practice guidelines, including multiple medications that impact vascular function (i.e., statins, low-dose aspirin, clopidogrel, beta-blockers).

Moreover, the impact of these medications (most notably aspirin which modifies prostanoid levels) on CYP-mediated eicosanoid metabolism has not been rigorously evaluated in preclinical models or humans, and thus remains unknown. Therefore, we cannot elucidate whether the observed relationships were modified by medication use. Although our adjusted and stratified analyses showed similar associations between biomarkers of CYP-mediated eicosanoid metabolism and vascular function phenotypes irrespective of medication use, evaluation of the direct impact of medication use on EET and 20-HETE levels, as well as the modifying effect of medication use on the observed eicosanoid-vascular function relationships, is beyond the scope of the current investigation and requires further study. Importantly, since pharmacologic agents in development that increase EETs (sEH inhibitors) or decrease 20-HETE (CYP ω-hydroxylase inhibitors) would most likely be used as a secondary prevention strategy for CAD, in addition to these standard of care medications, our study was designed to specifically characterize associations between biomarkers of CYP-mediated eicosanoid metabolism and vascular function in a population of stable, appropriately treated patients with established CAD. These data lay a foundation for the rational design of subsequent studies that aim to directly evaluate the effects of novel therapies that decrease 20-HETE and increase EET levels on vascular function in patients with established CAD.

Conclusions

This cross-sectional analysis demonstrates that higher circulating 20-HETE levels are associated with lower brachial artery FMD and higher CAMs and lower 14,15-EET:DHET ratios are associated with elevated MCP-1 and CAM levels in a population of patients with stable CAD. These findings suggest that enhanced CYP ω-hydroxylase and sEH metabolic function may predispose patients with established atherosclerotic cardiovascular disease to more advanced endothelial dysfunction and vascular inflammation despite current standards

of care. These findings lay a critical foundation for future clinical research in this area, including the rational design of interventional proof-of-concept studies that seek to define the vascular protective effects and safety of decreasing CYP-mediated 20-HETE biosynthesis and/or decreasing sEH-mediated EET hydrolysis in patients with established CAD.

Tables

Table 4.1. Correlation between vascular function phenotypes.

	FMD	CAM score	MCP-1	ENA-78	hs-CRP
FMD	==	-0.064 (0.526)	-0.101 (0.313)	-0.013 (0.901)	0.002 (0.984)
CAM score		==	0.120 (0.225)	0.115 (0.244)	0.071 (0.474)
MCP-1			==	0.017 (0.863)	-0.093 (0.345)
ENA-78				==	0.094 (0.338)
hs-CRP					==

Data presented as Pearson's correlation coefficient (p-value).

Table 4.2. Study population characteristics.

Characteristic	
N	106
Age (years)	58 ± 10
Female (%)	35 (33%)
African-American (%)	18 (17%)
Body mass index (kg/m^2)	30 ± 6
Obese (BMI ≥ 30 kg/m^2; %)	57 (54%)
Current smoker (%)	23 (22%)
Diabetes (%)	25 (24%)
Hypertension (%)	86 (81%)
Previous myocardial infarction	39 (37%)
Multivessel disease	70 (66%)
Recent revascularization procedure[a]	70 (66%)
Systolic blood pressure (mmHg)	136 ± 17
Diastolic blood pressure (mmHg)	80 ± 10
Total cholesterol (mmol/L)	4.04 (1.32)
LDL cholesterol (mmol/L)	2.20 (0.96)
HDL cholesterol (mmol/L)	1.22 (0.41)
Triglycerides (mmol/L)	1.11 (0.84)
ACE inhibitor or ARB (%)	66 (62%)
Beta-blocker (%)	89 (84%)
Statin (%)	99 (93%)
Aspirin (%)	103 (97%)
Clopidogrel (%)	83 (78%)

Data presented as mean ± standard deviation, median (interquartile range) or count (proportion).

ACE=angiotensin-converting enzyme, ARB=angiotensin receptor blocker, HDL=high density lipoprotein, LDL=low density lipoprotein.

[a]59/106 underwent a percutaneous coronary intervention and 11/106 underwent a coronary artery bypass grafting procedure between screening and the study visit.

Table 4.3. Correlation between biomarkers of CYP-mediated eicosanoid metabolism and vascular function.

CYP Eicosanoid	FMD	CAM Score	MCP-1	ENA-78	hs-CRP
20-HETE	**-0.255 (0.010)**	0.191 (0.051)	0.020 (0.843)	0.105 (0.287)	-0.090 (0.357)
Sum EETs	-0.095 (0.345)	-0.053 (0.599)	**-0.283 (0.003)**	0.115 (0.244)	0.045 (0.645)
14,15-EET:DHET	0.120 (0.229)	**-0.216 (0.027)**	**-0.252 (0.009)**	0.035 (0.723)	0.100 (0.307)

Data presented as Pearson's correlation coefficient (p-value).

Figures

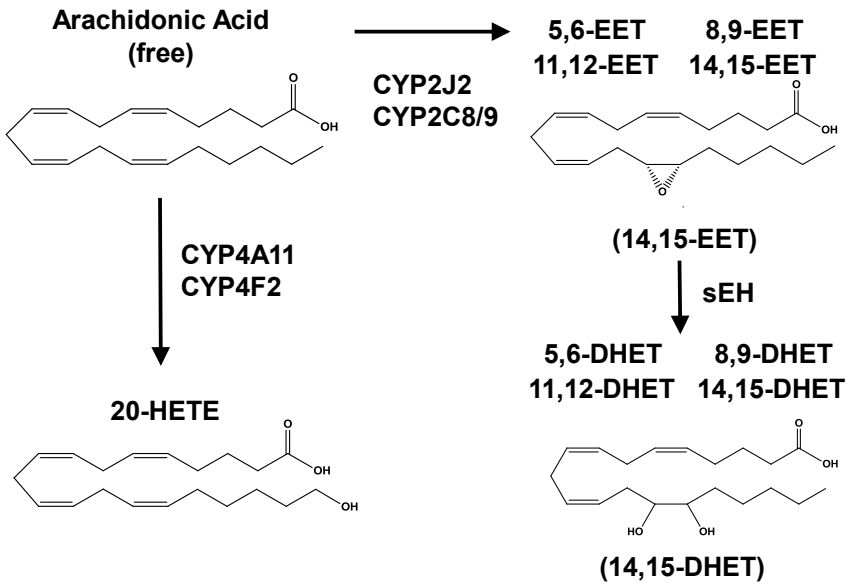

Figure 4.1. Overview of CYP-mediated arachidonic acid metabolism.

After release from the cell membrane by cytosolic phospholipase A_2, free arachidonic acid is metabolized by CYP ω-hydroxylases (CYP4A11 and CYP4F2) to 20-HETE or by CYP epoxygenases (CYP2J2, CYP2C8, and CYP2C9) to one of four EET regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET). The EETs (epoxides) are subsequently hydrolyzed by sEH to their corresponding DHET (diol) metabolites, which generally have less biological activity. Structures for the 14,15-EET regioisomer, the preferred EET substrate for sEH, and the 14,15-DHET metabolite are provided since the 14,15-EET:DHET (epoxide:diol) ratio is an established biomarker of sEH metabolic function.

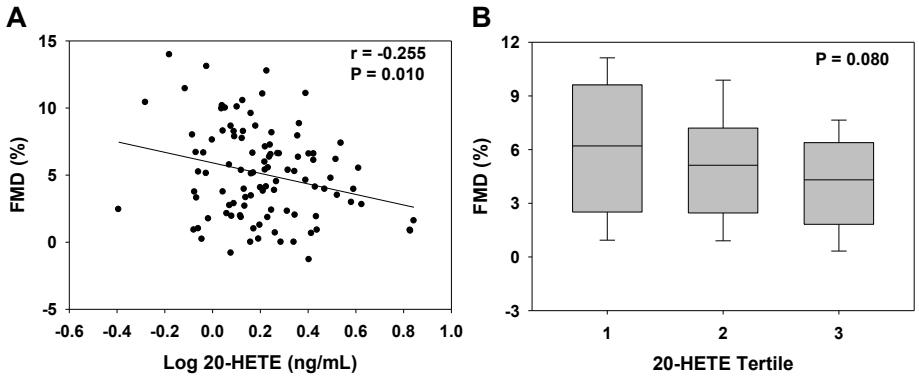

Figure 4.2. Association between 20-HETE and FMD.

(A) The correlation between 20-HETE and FMD is displayed. (B) The distribution of FMD across 20-HETE tertiles is displayed using box plots (tertile 1: 0.40-1.29 ng/mL, tertile 2: 1.30-1.85 ng/mL, tertile 3: 1.86-6.97 ng/mL). The horizontal line within the box represents the median, the edges of the box represent the 25th and 75th percentiles, and the error bars represent the 10th and 90th percentiles. The ANOVA p-value for the trend is provided.

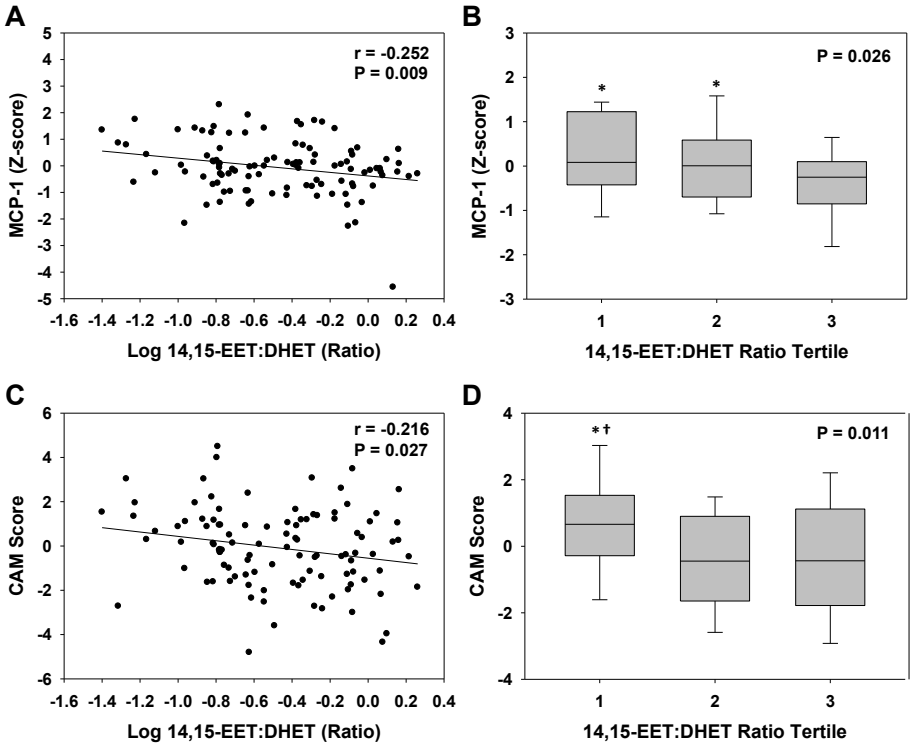

Figure 4.3. Association between 14,15-EET:DHET ratios and biomarkers of vascular inflammation.

The correlation between 14,15-EET:DHET ratio and (A) MCP-1 and (C) CAM score is displayed. The distribution of (B) MCP-1 and (D) CAM score across 14,15-EET:DHET ratio tertiles is displayed using box plots (tertile 1: 0.040-0189 ng/mL, tertile 2: 0.190-0.541 ng/mL, tertile 3: 0.542-1.82 ng/mL). The ANOVA p-value for the trend is provided. *P<0.05 versus tertile 3. †P<0.05 versus tertile 2.

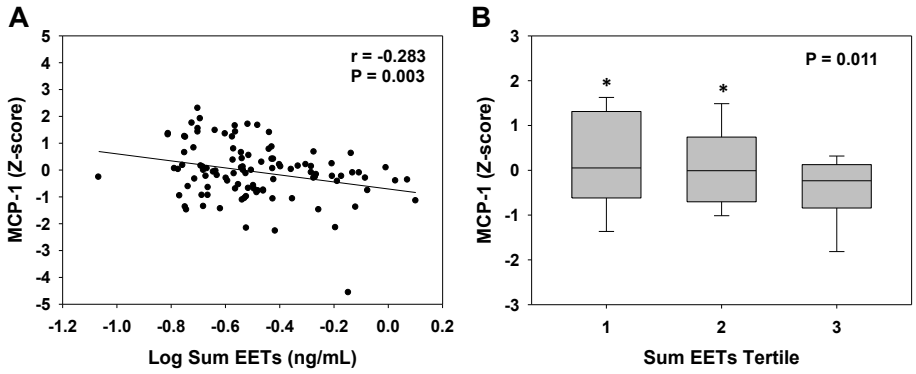

Figure 4.4. Association between sum EETs and MCP-1 levels.

(A) The correlation between sum EETs and MCP-1 is displayed. (B) The distribution of MCP-1 across sum EETs tertiles is displayed using box plots (tertile 1: 0.086-0.251 ng/mL, tertile 2: 0.252-0.375 ng/mL, tertile 3 0.376-1.26 ng/mL). The ANOVA p-value for the trend is provided. *P<0.05 versus tertile 3.

CHAPTER V

EVALUATION OF THE FUNCTIONAL ROLE OF THE CYTOCHROME P450 EPOXYGENASE PATHWAY IN FATTY LIVER DISEASE-ASSOCIATED INFLAMMATION

Introduction

Non-alcoholic fatty liver disease (NAFLD) affects approximately 30% of the general population in the United States, and nearly 70% of individuals with type 2 diabetes mellitus.[155] NAFLD begins with simple steatosis, and may progress to non-alcoholic steatohepatitis (NASH), and ultimately to advanced fibrosis and cirrhosis of the liver.[27] Although the progression from NAFLD to NASH is poorly understood, the development and progression of hepatic inflammation is a key pathological mediator in this transition and is associated with disease progression and the development of concomitant disease states including cardiovascular disease.[156, 157]

In the early stages of NAFLD, an imbalance between hepatocyte uptake and export of lipids leads to lipid accumulation within liver. Increased hepatic cholesterol levels activate toll-like receptors (TLRs) which signal through myeloid differentiation factor 88 (Myd88) to drive activation of nuclear factor-κB (NF-κB) mediated inflammatory responses.[158] NF-κB subsequently drives transcription of pro-inflammatory cellular adhesion molecules (which mediate monocyte adhesion to the endothelium), chemokines (which drive monocyte and macrophage recruitment), and cytokines (which propagate the local and systemic inflammatory response). Activation of the hepatic inflammatory response leads to monocyte activation and macrophage infiltration into the liver, ultimately causing fibrosis and hepatic injury.[159] Furthermore, this pathologic process drives systemic inflammation, and leads to

endothelial activation and the subsequent development of atherosclerosis.[26] Consistent with the pathological link between hepatic inflammation and atherosclerosis, the high-fat/high-cholesterol "atherogenic" diet model of steatohepatitis induces dyslipidemia, hepatic inflammation, and fibrosis, through an innate immune-mediated mechanism, which precedes the development of atherosclerosis.[160, 161]

Cytochrome P450 (CYP) enzymes are abundantly expressed in the liver where they catalyze the oxidative biotransformation of most exogenous drugs.[64] In addition, certain CYP isoforms are expressed in extrahepatic tissues and metabolize endogenous substrates including fatty acids, steroids, and retinoids to biologically active molecules.[65] Notably, CYP epoxygenase enzymes from the CYP2C and CYP2J subfamilies are highly expressed in endothelial cells and the liver, where they metabolize arachidonic acid to the biologically active 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid (EET) regioisomers;[66] however, EETs are rapidly hydrolyzed by soluble epoxide hydrolase (sEH) to their corresponding DHETs, which have much less biological activity.[66] Previous studies have shown that hepatic CYP epoxygenase expression and metabolic activity is suppressed in response to lipopolysaccharide (LPS) induced inflammation in a time-dependent manner, an acute inflammation model driven by innate immune system activation through TLR4.[162] In addition, increased endothelial EET biosynthesis, or decreased global sEH mediated EET hydrolysis, attenuates NF-kB activation and the acute vascular and systemic inflammatory response to LPS.[76, 77]

Collectively, these studies demonstrate that hepatic CYP epoxygenase activity is suppressed in response to activation of the innate immune system, and potentiation of the CYP epoxygenase pathway attenuates innate immune-dependent acute vascular and systemic inflammatory responses *in vivo*. However, the functional relevance of the CYP epoxygenase pathway in the development and progression of fatty liver disease-associated sustained inflammation has not been rigorously evaluated. Therefore, the objective of our

study was to 1) investigate the contribution of innate immune signaling to induction of steatohepatitis by the atherogenic diet, 2) characterize the effect of atherogenic diet-induced steatohepatitis on CYP epoxygenase expression and EET biosynthesis, and 3) determine if genetic and/or pharmacologic potentiation of the CYP epoxygenase pathway attenuates fatty liver disease-associated hepatic inflammation and injury.

Materials and Methods

Animals

Mice with targeted disruption of *Ephx2* (*Ephx2*$^{-/-}$) were rederived and backcrossed onto a C57BL/6 genetic background for more than 10 generations at the National Institute of Environmental Health Sciences (NEHS/NIH), as described.[73] Transgenic mice that express the human CYP2J2 enzyme in endothelial cells downstream of the murine *Tie2* promoter (*CYP2J2*-Tr) were developed on a C57BL/6 background as previously described.[163] We have previously reported significantly higher endothelial EET biosynthesis and circulating EET levels in *CYP2J2*-Tr mice compared to wild-type (WT) littermates, consistent with CYP epoxygenase overexpression.[77, 163] All experiments with *CYP2J2*-Tr mice used transgenic mice from multiple founder lines and WT littermates were included as controls; additional age-matched WT C57BL/6 mice and *Myd88*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were male, 8-20 weeks of age at the initiation of the experiments, had free access to food and water and were housed in temperature and humidity controlled rooms using a 12 hour light/dark cycle. All studies were completed in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*, and were approved by the Institutional Animal Care and Use Committee at the University of North Carolina-Chapel Hill (UNC).

Experimental Protocol

Mice were fed *ad libitum* an atherogenic (ATH) diet[164, 165] containing 40% kilocalories from fat, 1.25% cholesterol and 0.5% cholic acid (D12109c, Research Diets Inc., New Brunswick, NJ) or a standard chow (STD) diet containing 14% kilocalories from fat and 0.02% cholesterol (ProLab RMH 3000, PMI Nutrition International, Brentwood, MO). An initial pilot experiment was conducted to assess the relative induction of hepatic inflammation following two, four, or eight weeks of atherogenic diet administration. All subsequent studies were conducted over four weeks to evaluate the effects of 1) atherogenic diet feeding on CYP-mediated eicosanoid metabolism; 2) abrogation of signaling through Myd88 on atherogenic diet induction of hepatic inflammation and dysregulation of CYP-mediated eicosanoid metabolism; and, 3) CYP epoxygenase pathway potentiation on atherogenic diet induced hepatic inflammation and injury.

A subset of WT mice administered the atherogenic diet were concurrently administered the sEH inhibitor *trans*-4-[4-(3-adamantan-1-ylureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB, kindly provided by Dr. Bruce Hammock, University of California-Davis) or vehicle (0.5% polyethylene glycol 400 [PEG 400]) in drinking water for the four week duration of the experiment.[166] *t*-AUCB was dissolved in PEG 400 then diluted in tap water to a final concentration of 10 mg/L (*t*-AUCB group) or 50 mg/L (high-dose [HD]-*t*-AUCB group) in 0.5% PEG 400 and provided *ad libitum.*

Four independent studies were completed with 6-14 mice per group in each experiment plus 3-6 WT mice fed the standard diet (control group). Aggregate results are presented with a total of 19 WT mice fed the standard diet, 45 WT mice fed the atherogenic diet, 24 *Ephx2*$^{-/-}$ mice, 15 *CYP2J2*-Tr, 14 mice administered standard dose *t*-AUCB (10 mg/L) and 14 mice administered high-dose *t*-AUCB (50 mg/L). At study termination, mice were euthanized by CO_2 asphyxiation. Blood was collected via cardiac puncture, plasma was separated by

centrifugation and liver tissue was harvested and snap-frozen in liquid nitrogen. Plasma and tissue were stored at -80°C pending analysis.

Biochemical Analysis

Plasma total cholesterol and alanine aminotransferase (ALT) levels were quantified using a Vitros 350 automated chemical analyzer (Ortho-Clinical Diagnostics, Rochester, NY) at the UNC Animal Clinical Laboratory Core Facility. Total cholesterol and triglyceride levels were quantified in homogenized liver tissue using the Biovision Cholesterol Ester Quantification Kit II and the Biovision Triglyceride quantification Kit, respectively, according to the manufacturer's instructions (Biovision Incorporated, Milpitas, CA).

Formation of CYP-Derived Eicosanoids in Liver Microsomes

Microsomal fractions were isolated from hepatic tissue as previously described.[162] Frozen liver was homogenized in 0.25 M sucrose-10 mM Tris HCl buffer (pH 7.5) containing protease inhibitors. The resulting homogenates were centrifuged at 4°C at 2,570 *g* for 20 minutes followed by 10,300 *g* for 20 minutes; supernatants were collected and centrifuged at 100,000 *g* at 4°C for 90 minutes, and the resulting microsomal pellets were resuspended in 50 mM Tris-1 mM DTT-1 mM EDTA buffer (pH 7.5) containing 20% glycerol. Microsome protein concentrations were determined using the BCA method.[167] As previously described, 300 µg microsomal protein was diluted 10-fold with incubation buffer (0.12 M potassium phosphate buffer containing 5 mM magnesium chloride) then incubated with 50 µM arachidonic acid in 1 mL of incubation buffer.[162] Incubations were completed at 37°C for 20 minutes; reactions were initiated by adding 1 mM NADPH and terminated by placing the samples ice. The incubation procedure was repeated using a fresh aliquot of microsomal protein in the presence of 5 µM *t*-AUCB. Following termination of the reaction 12.5 ng of internal standard (20-HETE-d6) was added and metabolites were extracted with diethyl

ether, dried under a stream of nitrogen gas and reconstituted in 80% methanol in deionized water for analysis. Incubations were performed under saturating concentrations of arachidonic acid, and preliminary incubations demonstrated formation was linear with respect to incubation time and microsomal protein quantity. Under these saturating conditions, formation rates reflect the amount of biologically active protein and are significantly correlated with CYP mRNA and protein levels.[162, 168]

Metabolites of arachidonic acid were then quantified by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), as described.[162] Analytes were separated using a UPLC BEH C-18 1.7 μM (2.1 x 100 mm) reverse-phase column (Waters Corporation, Milford, MA). Mass spectrometry was performed using a TSQ Quantum Ultra triple-quadrupole mass spectrometer coupled with heated electrospray ionization operated in negative selective reaction monitoring mode (Thermo Fisher Scientific, San Jose, CA). Data were acquired and analyzed using Xcaliber software version 2.0.6 (ThermoFinnigan, San Jose, CA) and metabolite concentrations were calculated from a standard curve and expressed relative to the standard diet treated group.

Direct Quantification of CYP-Derived Eicosanoids in Plasma and Liver

Arachidonic acid metabolites from the CYP epoxygenase pathway (8,9-, 11,12- 14,15-EET, 5,6-, 8,9- 11,12-, 14,15-DHET), CYP ω-hydroxylase pathway (20-hydroxyeicosatetraenoic acid [HETE]) and linoleic acid metabolites from the CYP epoxygenase pathway (12,13-epoxyoctadecenoic acid [EpOME] and 12,13-dihydroxyoctadecenoic acid [DHOME]) were quantified in plasma and tissue as previously described.[145] Plasma (200 uL) was diluted in 250 uL of a 0.1% acetic acid/5% methanol solution containing 0.009 mM butylated hydroxytoluene (BHT) and spiked with 30 ng each of PGE2-d4, 10,11-DiHN, and 10,11-EpHep (Cayman Chemical, Ann Arbor, MI) which served as internal standards. Samples were loaded onto HyperSep Retain PEP SPE cartridges

(ThermoFisher Scientific, Waltham, MA) that were conditioned with 0.1% acetic acid/5% methanol. Columns were then washed twice with 0.1% acetic acid/5% methanol and dried for 10 minutes under a full vacuum. Samples were eluted into glass tubes containing 6 uL of 30% glycerol using 0.5 mL of methanol followed by two consecutive additions of 0.5 mL ethyl acetate.

Liver tissue (approximately 20 mg) was homogenized in 0.1% acetic acid/5% methanol solution containing 0.009 mM BHT and 100 µM *t*-AUCB (5% w/v). Internal standards (10,11-DiHN, and 10,11-EpHep) were added to glass tubes and liver lysates were vortexed with 2 ml of ethyl acetate for 10 minutes and the organic layer was removed and added to a glass collection tube containing 6 uL of 30% glycerol. The remaining lysate was vortexed with an additional 2 ml of ethyl acetate for 10 minutes and the organic layer was again added to the collection tube.

Metabolites were dried under a stream of nitrogen gas while heated to 37°C; tubes were purged with argon gas and stored at -80°C pending analysis. Samples were reconstituted using 50 uL of 30% ethanol, vortexed, and centrifuged at 2000 *g* for 2 minutes; 50 uL of the reconstituted sample was injected in triplicate. Liquid chromatography of extracted samples was performed with an Agilent 1200 Series capillary HPLC (Agilent Technologies, Santa Clara, CA). Separations were performed using a Phenomenex Luna C18(2) column (5 um, 150x1 mm, Phenomenex, Torrance, CA) while held at 40°C. Eicosanoid concentrations were quantified using a MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with negative mode electrospray ionization. The plasma 14,15-EET:DHET ratio was calculated as an *in vivo* biomarker of sEH metabolic function[73] and the sum of EET levels (8,9-, 11,12- 14,15-EET) in plasma were calculated as a biomarker of CYP epoxygenase pathway function as previously described.[163]

Gene Set Enrichment Analysis

Total RNA was isolated from homogenized tissue (n=4 per group) using the RNeasy mini-prep kit (Qiagen, Valencia, CA). Global gene expression analysis was conducted using the Agilent Whole Mouse Genome 4x44 multiplex array (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's protocol. Briefly, 1.65 micrograms of Cy3 labeled cRNAs were fragmented and hybridized for 17 hours in a rotating hybridization oven. Slides were then washed and scanned, data was acquired using the Agilent Feature Extraction software version 9.5 (Agilent Technologies) using 1-color defaults for all parameters. The resulting data were processed and analyzed using Genespring version 12.1 (Agilent Technologies). Only probes flagged "detected" in at least 75% of samples from at least one treatment group were included, resulting in 31,643 probes in the final dataset.

Gene set enrichment analysis (GSEA) Version 2.0 was used to determine if the arachidonic acid metabolism pathway is "enriched" in WT mice administered the atherogenic compared to WT mice administered the standard diet. Genes involved in the metabolism of arachidonic acid to biologically active eicosanoids were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG)[131] 'arachidonic acid metabolism pathway' for mice map00590, and limited to genes encoding proteins involved in arachidonic acid liberation from the cell membrane and metabolism.

Quantitative RT-PCR

Quantitative RT-PCR was performed by reverse transcribing RNA to cDNA using the ABI high-capacity cDNA reverse transcription kit (Applied Biosystems) with a reaction temperature of 25°C for 10 min followed by 37°C for 120 min. Quantitative RT-PCR reactions were performed in triplicate using the ABI 7300 Real-Time PCR system. Expression of hepatic *Tlr2, Tlr4, Cyp2c29, Cyp2c50, Cyp2j5, Ephx2, Saa1, Tnfα, Ccl2 Nfκb1,* and *Cd68* was quantified using Taqman® Assays on Demand (Applied Biosystems),

normalized to *Gapdh* (endogenous control) and expressed relative to the control group using the $2^{-\Delta\Delta Ct}$ method.[169]

ELISA

Liver tissue was homogenized in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X, 1 mM NaF, 0.25% Na deoxycholate and protease inhibitors) and the S9 fraction was separated by centrifugation. Protein concentrations of the homogenate were quantified using the BCA method.[167] Monocyte chemoattractant protein-1 (MCP-1), and vascular cellular adhesion molecule-1 (VCAM-1) protein levels were quantified in liver homogenates using the mouse CCL2/JE/MCP-1 and VCAM-1/CD106 Quantikine® ELISA kits (R&D Systems, Minneapolis, MN, USA), respectively, after loading equal amounts of protein into each well. Concentrations were normalized to mg of liver protein and expressed relative to the atherogenic diet group. Plasma concentrations of MCP-1 were quantified using the mouse CCL2/JE/MCP-1 Quantikine® ELISA kit (R&D Systems) following a four-fold dilution.

Histology and Immunohistochemistry

Liver tissue (left lobe) was fixed in 4% paraformaldehyde for 24 hours, processed, embedded in paraffin, and cut into 5 μm sections. Immunohistochemistry was performed by treating slides with pH 6.0 sodium citrate buffer (Dako North America, Carpinteria, CA) and endogenous peroxide was quenched by placing slides in a solution of 3% H_2O_2 for 15 minutes. Sections were blocked using 0.25% casein in PBS (Dako North America) then incubated with a rabbit anti-F4/80 polyclonal antibody at a 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a goat anti-rabbit secondary antibody at a 1:500 dilution (Jackson Immunoresearch, West Grove, PA). Antibody binding was detected using Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and visualization was

performed with 3,3-diaminobenzidine (Vector Laboratories). Sections were then counterstained with hematoxylin, dehydrated, and mounted. Digital images were acquired with the ScanScope CS slide capture device (Aperio, Vista, CA) and analyzed using ImageScope Version 11.1 (Aperio).

Macrophage infiltration was quantified by counting the number of inflammatory foci per 10x field, as described.[170] All analyses were performed in duplicate by the same individual who was blinded to treatment group. Three fields were analyzed in each of three sections which were cut 200 μm apart; the number of foci were then summed and averaged to obtain an estimate for each animal.

The presence and extent of NAFLD was evaluated on hematoxylin and eosin (H&E) stained slides according to a standardized histological scoring system (NAFLD Activity Score) that evaluates the presence and severity of steatosis, lobular inflammation, hepatocyte ballooning, and fibrosis.[171] Steatosis (the accumulation of fatty deposits in the liver) is graded by quantifying the percentage of the surface area with fat droplets (0-3), hepatocyte ballooning is graded by the number of ballooned (i.e. enlarged) hepatocytes (0-2), and fibrosis is graded according to the extent and location of collagen deposition (0-4). Lobular inflammation, which is most the most relevant histological consequence of the atherogenic diet,[161] is graded according to the number of inflammatory infiltrates per 200x field as follows: 0 (no infiltrates); 1 (<2 infiltrates per field); 2 (2-4 infiltrates per field); and 3 (>4 infiltrates per field).

Statistical Analysis

Data were normalized to the atherogenic diet treated WT control group and pooled across experiments, unless otherwise indicated, and expressed as mean ± standard error of the mean (SEM). For continuous variables, rank-transformed mean values were compared using a one-way ANOVA followed by Fisher's LSD post hoc test with $p<0.05$ considered

statistically significant. Lobular inflammation score was analyzed by Chi-squared test with a post hoc logistic regression analysis. Correlation between continuous variables was evaluated using Spearman's rank correlation. All statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC).

Results

Section 1: Characterization of the CYP Epoxygenase Pathway in NAFLD/NASH

Characterization of the Atherogenic Diet Model of NAFLD/NASH

Inflammation was evaluated in liver tissue following two, four, or eight weeks of atherogenic diet administration by quantifying mRNA expression of *Saa1,* (serum amyloid A, an acute phase reactant), *Ccl2*, (MCP-1, a chemokine that recruits monocytes to the vessel wall), and *Cd68* (a glycoprotein highly expressed on macrophages). Hepatic *Saa1*, *Ccl2*, and *Cd68* were significantly induced at two, four, and eight weeks (Figure 5.1A-C). Similarly, plasma ALT levels, a biomarker of hepatic injury, were significantly increased after two, four, and eight weeks of atherogenic diet administration (Figure 5.1D). Since inflammation and hepatic injury were not further increased with longer duration administration of the atherogenic diet, the four-week time-point was utilized for all follow-up studies.

Plasma total cholesterol (Figure 5.2A), hepatic total cholesterol (Figure 5.2B), and hepatic triglyceride levels (Figure 5.2C) were significantly higher in mice administered the atherogenic diet compared to mice administered the standard chow diet. Body weight did not change significantly from baseline (-0.18±0.21 grams, $p=0.397$) in response to the atherogenic diet; however, mice administered the standard diet experienced modest weight gain (1.16±0.16 grams, $p<0.001$). Collectively, these data demonstrate that the atherogenic diet model induces hepatic lipid accumulation, inflammation, macrophage infiltration and

injury, which are key drivers of the development and progression of NAFLD/NASH in humans.

Differential Expression of the Arachidonic Acid Metabolism Pathway in NAFLD/NASH

Gene set enrichment analysis was utilized to determine if the arachidonic acid metabolism pathway was enriched in WT mice administered the atherogenic diet. Of the 82 genes in the arachidonic acid metabolism pathway, 67 (82%) were included on the microarray and passed our quality control standards (indicating that they were expressed in 75% samples within at least one treatment group). The arachidonic acid metabolism pathway was significantly enriched in mice administered the atherogenic diet ($p<0.001$). The core enrichment subset, which is the subset of genes accounting for the observed signal, was down-regulated in mice administered the atherogenic diet compared to mice administered the standard chow diet (Figure 5.3), indicating that relative to global gene expression changes, the arachidonic acid metabolism pathway was suppressed in mice administered the atherogenic diet. Twenty-two of the 28 (79%) genes included in the core enrichment subset were CYP transcripts, and 10 of the 22 CYPs (46%) were from the CYP2C and CYP2J subfamilies, which are CYP epoxygenases.

CYP Eicosanoid Metabolism

Administration of the atherogenic diet for four weeks significantly decreased plasma and liver EET levels (Figure 5.4A and 5.4D), which were highly correlated ($r_s=0.876$, $p<0.001$). In addition, formation rates of sum EETs and sum EETs+DHETs were significantly suppressed in liver microsomes from mice fed the atherogenic diet compared to mice fed the standard chow diet both with and without *t*-AUCB in the incubation (Figure 5.4B-C and 5.4E-F). Consistent with suppression of hepatic CYP epoxygenase activity, liver *Cyp2c29* mRNA levels, were significantly suppressed in mice administered the atherogenic diet (Figure

5.5A). Similar results were observed with liver mRNA levels of the *Cyp2c50*, *Cyp2c55*, and *Cyp2j5* epoxygenases (data not shown). However, no significant differences in hepatic *Ephx2* mRNA levels (Figure 5.5B) were observed.

In contrast to the atherogenic diet induced suppression of the CYP epoxygenase pathway, plasma 20-HETE levels (0.45±0.15 vs. 0.45±0.05 ng/ml, p=0.940), liver 20-HETE levels (9.7±4.6 vs. 6.7±1.4 ng/g, p=0.689) and liver microsome 20-HETE formation rates (342±14 vs. 374±19 pmol/mg protein/minute, p=0.689) were similar in mice fed the standard and atherogenic diets, respectively.

Role of the Innate Immune System in Fatty Liver Disease-Associated Inflammation

Hepatic expression of *Tlr2* and *Tlr4* was significantly induced in mice fed the atherogenic diet for four weeks compared to mice fed the standard chow diet (Figure 5.6), indicating that the innate immune system is activated in response to atherogenic diet feeding. Administration of the atherogenic diet significantly increased plasma MCP-1, hepatic MCP-1 and hepatic VCAM-1 protein levels, and macrophage infiltration into liver tissue, all of which were attenuated in *Myd88*$^{-/-}$ mice (Figure 5.7 and Figure 5.8).

Consistent with the results of our previous experiment, administration of the atherogenic diet significantly suppressed plasma and liver sum EET levels in WT mice; a similar level of suppression was observed in *Myd88*$^{-/-}$ mice (Figure 5.9A and 5.9C). The 14,15-EET:DHET ratio was not significantly different in WT or *Myd88*$^{-/-}$ mice fed the atherogenic diet compared to standard diet fed mice in either plasma or liver (Figure 5.9B and 5.9D). Consistent with suppression of hepatic CYP epoxygenase activity, liver *Cyp2c29* mRNA levels were significantly suppressed in WT mice administered the atherogenic diet (37% of control), and were partially restored in *Myd88*$^{-/-}$ mice fed the atherogenic diet (54% of control, Figure 5.9E). However, *Ephx2* mRNA levels were not significantly different in WT or *Myd88*$^{-/-}$ mice administered the atherogenic diet (Figure 5.9F).

Administration of the atherogenic diet increased hepatic mRNA expression of *Tlr4, Nfκb1,* and *Tnfα*; induction of *Tlr4, Nfκb1,* and *Tnfα* was significantly attenuated in *Myd88*$^{-/-}$ mice, but remained significantly higher in *Myd88*$^{-/-}$ mice compared to WT mice fed the standard diet (Figure 5.10A, 5.10D, and 5.10G). Hepatic *Tlr4, Nfκb1, and Tnfα* mRNA levels each exhibited a significant inverse correlation with hepatic *Cyp2c29* expression and sum EET levels in liver tissue (Figure 5.10). Similar correlations were observed between hepatic *Tlr4, Nfκb1,* and *Tnfα* mRNA levels and *Cyp2c50*, *Cyp2c55*, and *Cyp2j5* mRNA levels (data not shown).

Summary (Section 1)

The atherogenic diet model of NAFLD/NASH increases circulating and hepatic cholesterol levels and hepatic triglycerides, activates the innate immune system, and induces hepatic inflammation and injury. In addition, hepatic expression of CYP epoxygenase enzymes, hepatic EET biosynthesis, and hepatic and plasma EET levels are significantly suppressed in response to atherogenic diet feeding, and this suppression is correlated with activation of the innate immune response. However, changes in hepatic 20-HETE biosynthesis, and hepatic and plasma 20-HETE levels were not altered in mice administered the atherogenic diet. Moreover, abrogation of signaling through Myd88 attenuates, but does not completely reverse, the induction of hepatic inflammation, and does not restore hepatic CYP epoxygenase expression or hepatic and plasma EET levels.

<u>Section 2:</u> Potentiation of the CYP Epoxygenase Pathway in NAFLD/NASH

Genetic Disruption and Pharmacologic Inhibition of sEH

In order to evaluate sEH inhibition as a therapeutic target to attenuate fatty liver disease-associated inflammation, we administered the atherogenic diet for four weeks and quantified

plasma and liver eicosanoid levels in *Ephx2*$^{-/-}$ mice, WT mice administered *t*-AUCB at 10 mg/L (*t*-AUCB group) and WT mice administered high-dose *t*-AUCB at 50 mg/L in drinking water (HD *t*-AUCB group). Consistent with inhibition of sEH mediated EET hydrolysis, the 14,15-EET:DHET ratio and the 12,13-EpOME:DHOME ratio were significantly higher in *Ephx2*$^{-/-}$ mice and in each of the *t*-AUCB treated groups compared to WT mice fed the atherogenic diet in both plasma and liver (Figure 5.11A-B and 5.11D-E). In addition, inhibition of sEH resulted in a significant increase in sum EET levels in both plasma and liver tissue in *Ephx2*$^{-/-}$ mice and in each of the *t*-AUCB treated groups compared to WT mice fed the atherogenic diet (Figure 5.11C and 5.11F). However, plasma epoxide:diol ratios and sum EETs were higher in the *Ephx2*$^{-/-}$ mice compared to each of the *t*-AUCB treated groups, indicating that pharmacologic inhibition did not decrease epoxide hydrolysis to the degree observed in *Ephx2*$^{-/-}$ mice. However, these differences were less pronounced in liver. The increase in EET levels was similar for both the 8,9- and 14,15-EET regioisomers; however, significant differences were not observed across groups for the 11,12-EET regioisomers (Figure 5.12). Plasma and liver total cholesterol levels were significantly increased in response to atherogenic diet administration ($p<0.001$ vs. standard diet); however, sEH inhibition did not impact cholesterol levels (data not shown).

Expression of Inflammatory Mediators

Compared to the standard chow diet, administration of the atherogenic diet significantly increased plasma MCP-1 (2.3±0.3-fold), hepatic MCP-1 (8.6±1.5-fold), and hepatic VCAM-1 (4.4±0.5-fold) protein levels (Figure 5.13A-C, $p<0.001$ vs. standard diet group for each). The induction of chemokine and CAM expression was significantly attenuated in the *Ephx2*$^{-/-}$ mice ($P<0.01$ vs. atherogenic diet group for each), and plasma MCP-1 levels were significantly attenuated in the high-dose *t*-AUCB group ($p=0.037$). However, no significant differences were observed in the *CYP2J2*-Tr and *t*-AUCB treated groups (Figure 5.13A-C).

Hepatic Macrophage Infiltration

Administration of the atherogenic diet resulted in significant infiltration of macrophages into liver tissue as demonstrated by F4/80 staining (Figure 5.14). Mice fed the atherogenic diet had an average of 3.4±0.41 inflammatory foci per 10x field compared to only 0.14±0.02 inflammatory loci per field in the standard diet fed mice ($p<0.001$). Macrophage infiltration into liver tissue was significantly attenuated in the *Ephx2*$^{-/-}$ mice (2.0±0.50 foci) compared to the atherogenic diet group ($p=0.022$). In contrast, pharmacologic inhibition of sEH and endothelial overexpression of CYP2J2 did not significantly attenuate macrophage infiltration (Figure 5.14G).

Pathological Assessment of Hepatic Damage

NAFLD severity was assessed by quantifying steatosis, lobular inflammation, hepatocyte ballooning and fibrosis.[171] In response to four weeks of atherogenic diet administration, steatosis was present in <5% of all samples, hepatocyte ballooning was absent in all samples, and fibrosis was minimal or absent in all samples. Therefore, our analysis focused on lobular inflammation, which was noted in the majority of mice administered the atherogenic diet (Figure 5.15A-G, Table 5.1).[171] Chi-square analysis of lobular inflammation indicated atherogenic diet administration induced a significant increase in lobular inflammation ($p<0.001$). A post hoc ordinal logistic regression analysis demonstrated that *Ephx2*$^{-/-}$ mice were significantly more likely to have lower levels of lobular inflammation than WT mice administered the atherogenic diet ($p=0.011$); however no other treatment groups were significantly different than the atherogenic diet group (Table 5.1). When analyzed as a continuous variable, the atherogenic diet group (1.44±0.17) had a significantly higher inflammation score than the standard diet group (0.00±0.00, $p<0.001$), and *Ephx2*$^{-/-}$ mice (0.71±0.19) had significantly lower inflammation scores than WT mice fed the atherogenic

diet (p=0.006). Similar to the ordinal logistic regression analysis, no other treatment groups were significantly different than the atherogenic diet group (Figure 5.15G)

Hepatic Injury

Plasma ALT levels, a biomarker of hepatic injury and necrosis, increased 6.2±0.87-fold in mice fed the atherogenic diet compared to mice fed the standard diet, and *Ephx2*$^{-/-}$ mice had significantly lower plasma ALT levels than WT atherogenic diet fed mice (Figure 5.16, p=0.017 vs. the atherogenic diet group); however the *CYP2J2*-Tr mice (p=0.480), and mice administered *t*-AUCB (p=0.339) and high-dose *t*-AUCB (p=0.275) were not significantly different than the atherogenic diet group.

Summary (Section 2)

Our analysis of the therapeutic effects of CYP epoxygenase pathway potentiation in fatty liver disease-associated inflammation demonstrated that genetic disruption of sEH significantly attenuated the induction of key inflammatory mediators, macrophage infiltration into liver and liver injury in response to atherogenic diet administration. However, pharmacologic inhibition of sEH and endothelial overexpression of CYP2J2 did not produce a similar anti-inflammatory effect.

Discussion

Potentiation of the CYP epoxygenase pathway elicits potent anti-inflammatory effects in preclinical models of acute and chronic vascular inflammation, and has been proposed as an anti-inflammatory therapeutic strategy for the treatment of inflammatory diseases.[74] Chronic hepatic inflammation is a key pathologic mediator in the progression of NAFLD; however, the contribution of CYP-mediated eicosanoid metabolism to fatty liver disease-

associated inflammation has not been rigorously evaluated in preclinical models of NAFLD/NASH. This study is the first to demonstrate that 1) expression of hepatic CYP epoxygenase enzymes, hepatic EET biosynthesis and circulating EET levels are significantly suppressed in an atherogenic diet model of NAFLD/NASH and 2) genetic disruption of sEH restores hepatic and systemic EET levels and attenuates atherogenic diet induced hepatic inflammation and injury. These findings demonstrate that suppression of EET biosynthesis is a key pathological consequence of NAFLD/NASH, and therapeutic restoration of EET levels is an anti-inflammatory therapeutic strategy with potential utility for the treatment of fatty liver disease-associated inflammation. Future studies will seek to define the mechanism underlying the suppression of EET biosynthesis in the presence of NAFLD/NASH. In addition, the observed differences between genetic disruption and pharmacologic inhibition of sEH highlight the need for rigorous characterization of the mechanisms by which EETs attenuate hepatic inflammation, and to evaluate the utility of therapeutic strategies that increase EETs as a treatment for NAFLD/NASH.

Previous studies have demonstrated that inflammatory stimuli suppress hepatic CYP-mediated xenobiotic metabolism.[172] In addition, we have reported that the hepatic EET biosynthesis is suppressed in a LPS model of acute inflammation and a high-fat diet model of insulin resistance.[162, 173] Our microarray analysis demonstrated that, relative to global gene expression changes, the arachidonic acid metabolism pathway (including numerous CYP epoxygenase enzymes) is significantly suppressed in response to atherogenic diet administration. Moreover, we observed suppression of hepatic *Cyp2c29*, *Cyp2c50*, *Cyp2c55*, and *Cyp2j5*, the most abundant CYP epoxygenases in mouse liver.[162] This finding suggests that suppression of CYP epoxygenase enzymes is an important event in the pathogenesis of NAFLD/NASH, and is consistent with a previous report demonstrating that expression of hepatic *Cyp2c29* is suppressed in response to choline- and folate-deficient diet administration, a model of NAFLD/NASH that induces hepatic steatosis and

inflammation.[174] Consequently, we investigated the effects of atherogenic diet administration on CYP-mediated eicosanoid metabolism in WT mice. We observed that CYP epoxygenase pathway activity is suppressed in liver microsomes following four weeks of atherogenic diet administration. In addition, both plasma and liver EET levels were suppressed in mice fed the atherogenic diet compared to mice fed a standard chow diet. Our study is the first to demonstrate that EET biosynthesis in liver microsomes, and hepatic and plasma EET levels, are suppressed in the presence of NAFLD/NASH. Collectively, these data suggest that suppression of hepatic CYP epoxygenase-mediated EET biosynthesis is a key pathological consequence of NAFLD/NASH.

Previous studies have demonstrated that LPS induced inflammation suppresses hepatic CYP epoxygenase expression *in vivo*.[162] In addition, inflammatory cytokines including IL-1, IL-6 and TNFα suppress CYP expression in hepatocytes, and cytokine-mediated suppression of CYPs is dependent on NF-κB activation.[175, 176] Despite this clear effect, the contribution of specific nuclear receptors known to regulate CYP expression on the cytokine-mediated suppression of CYPs appears isoform and species specific and model dependent, suggesting that upstream activation of the innate immune response is the most important factor in CYP suppression. Therefore, we hypothesized that abrogation of Myd88-dependent activation of NF-κB would prevent the atherogenic diet induced suppression of CYP epoxygenase expression and EET biosynthesis. Genetic disruption of *Myd88* attenuated the atherogenic diet induced increase in plasma and liver inflammatory biomarkers and macrophage infiltration into liver demonstrating a significant inhibition, but not complete abrogation, of the inflammatory response. However, despite a partial attenuation of *Cyp2c29* suppression in liver tissue, disruption of *Myd88* did not restore the atherogenic diet induced suppression of plasma or hepatic EETs.

To further investigate the mechanism underlying the atherogenic diet induced suppression of the CYP epoxygenase pathway, we quantified mRNA expression of *Tlr4,*

Nfκb1, and *Tnfα*. Induction of *Tlr4, Nfκb1,* and *Tnfα* transcript levels were attenuated in *Myd88*$^{-/-}$ mice, but remained significantly elevated compared to WT mice administered the standard diet. Interestingly, a strong inverse correlation was observed between *Tlr4, Nfκb1,* and *Tnfα* expression and CYP epoxygenase expression. Moreover, a significant inverse association was also observed between hepatic *Tlr4, Nfκb1,* and *Tnfα* expression and EET levels in liver. Collectively, these data suggest that innate immune system activation is associated with suppression of the CYP epoxygenase pathway, and that the mechanism driving CYP epoxygenase suppression is, at least in part, independent of signaling through Myd88. Future studies directly evaluating the role of Myd88-independent activation of NF-κB in atherogenic diet induced CYP epoxygenase pathway suppression are warranted.

CYP derived EETs have potent anti-inflammatory effects in preclinical models of NF-κB mediated vascular inflammation.[75, 77] In addition, recent reports indicate that sEH inhibition may attenuate the development of insulin resistance and hepatic steatosis in response to a high-fat diet.[177, 178] However, the anti-inflammatory effects of CYP epoxygenase pathway potentiation have not been rigorously evaluated in an atherogenic diet model of NAFLD/NASH, which induces hepatic inflammation via an innate immune system dependent mechanism. Since our studies demonstrated that suppression of CYP mediated EET biosynthesis is a pathological consequence of atherogenic diet feeding, we hypothesized that restoring CYP epoxygenase pathway activity by increasing EET biosynthesis via endothelial CYP2J2 overexpression, or globally decreasing EET hydrolysis by targeted disruption of *Ephx2* or pharmacologic inhibition of sEH, would attenuate atherogenic diet induced hepatic inflammation. Consistent with our hypothesis and the anti-inflammatory effects of EETs, genetic disruption of sEH attenuated macrophage infiltration into liver tissue, hepatic expression of inflammatory mediators, and hepatic injury in response to atherogenic diet feeding.

Surprisingly, endothelial CYP2J2 overexpression and pharmacologic inhibition of sEH did not result in an anti-inflammatory effect similar to that observed in the *Ephx2*$^{-/-}$ mice. Previous studies have shown that endothelial CYP2J2 overexpression and genetic disruption of sEH each attenuate the LPS induced inflammatory response in lung tissue to a similar degree,[77] and pharmacologic inhibition of sEH attenuates the LPS induced increase in circulating inflammatory biomarkers.[76] Similarly, administration of *t*-AUCB attenuated hepatic induction of TNFα in response to high fat diet feeding for 16 weeks.[177] However, while genetic disruption of sEH attenuated the induction of inflammation in response to 8 weeks of high fat diet feeding, administration of *t*-AUCB to WT mice for the final 4 weeks of the experiment had no effect.[177] Interestingly, similar differences in phenotypic response to genetic disruption and pharmacologic inhibition of sEH have also been reported in a mouse model of hypoxia-induced pulmonary hypertension.[179] Collectively, these data suggest that the anti-inflammatory effects of sEH inhibition may be model dependent, and that pharmacologic inhibition and genetic disruption of sEH may not always produce consistent phenotypes.

The atherogenic diet model increases circulating cholesterol levels and induces hepatic and systemic inflammation; therefore, therapies that reduce plasma cholesterol levels may have an anti-inflammatory effect. Of note, sEH is a bifunctional enzyme with both an epoxide hydrolase and lipid phosphatase domain.[180] Previous studies have shown that genetic disruption of sEH, which abolishes activity in both domains, results in lower plasma cholesterol levels, whereas pharmacologic inhibition of sEH, which inhibits only hydrolase activity, has no effect on cholesterol.[181] In the present study, neither genetic disruption nor pharmacologic inhibition of sEH had an effect on the atherogenic diet induced increase in plasma or hepatic cholesterol levels; therefore, it is unlikely that differences in cholesterol metabolism account for the differences in anti-inflammatory phenotypes. The differences in anti-inflammatory effect observed between genetic disruption and pharmacologic inhibition

of sEH may be secondary to insufficient inhibition of sEH with administration of *t*-AUCB. The 14,15-EET:DHET ratio was significantly increased 4.0±0.26-fold and 4.6±0.24-fold in mice administered *t*-AUCB at 10 mg/L and 50 mg/L, respectively, compared to mice fed the atherogenic diet only, which is similar to the level of sEH inhibition observed in previous studies.[166] In addition, sum EET levels were significantly higher in mice administered *t*-AUCB at both 10 mg/L and 50 mg/L compared to mice fed the atherogenic diet only, demonstrating inhibition of sEH led to a significant increase in circulating EETs. However, *Ephx2*$^{-/-}$ mice had significantly higher 14,15-EET:DHET ratio and sum EET levels in plasma compared to each of the *t*-AUCB groups, suggesting that *t*-AUCB at these doses did not inhibit EET hydrolysis to the same degree as *Ephx2*$^{-/-}$ disruption. In addition, high-dose, but not standard dose, *t*-AUCB administration significantly attenuated the induction of plasma MCP-1 levels, suggesting that more potent inhibition may be necessary to achieve an increase in EET levels (i.e. EET "dose") that is sufficient to produce an anti-inflammatory effect. These findings are consistent with the absence of an anti-inflammatory phenotype in the *CYP2J2*-Tr mice, which demonstrate only a modest ~1.5-fold elevation in circulating EETs despite significantly higher EET biosynthesis in isolated endothelial cells,[163] suggesting that higher EETs specifically in endothelial cells does not produce a high enough EET "dose" to attenuate macrophage infiltration into liver tissue.

Collectively, these findings suggest that the CYP epoxygenase pathway is an important regulator of atherogenic diet induced chronic hepatic inflammation. EETs exhibit anti-inflammatory, anti-apoptotic, and anti-fibrotic properties in preclinical models;[71, 74, 182] therefore the observed suppression of hepatic CYP epoxygenase pathway activity and circulating EET levels may be detrimental in the pathological progression of fatty liver disease. Indeed, *Ephx2*$^{-/-}$ mice fed an atherogenic diet had circulating EET levels greater than WT mice fed a standard diet, and exhibited significantly attenuated macrophage infiltration into liver tissue, lower hepatic and circulating mediators of inflammation, and

reduced hepatic injury, suggesting that restoration of the CYP epoxygenase pathway attenuates the development and progression of fatty liver disease. Importantly, these effects were independent of changes in circulating and hepatic cholesterol levels. Pharmacologic inhibition of sEH, however, did not produce a similar effect, suggesting the need for more potent inhibitors, which are currently in development,[183] or other therapeutic strategies that potentiate the effects of CYP-derived EETs. Additional studies are warranted to further investigate the mechanisms underlying the observed differences between genetic disruption and pharmacologic inhibition of sEH.

Our study has limitations that must be acknowledged. We evaluated the contribution of the CYP epoxygenase pathway to the regulation of fatty liver disease-associated inflammation following four weeks of atherogenic diet administration. The relatively short duration of our study has limitations as a model of human NAFLD/NASH, where chronic obesity leads to accumulation of fat within the liver (NAFLD) which eventually results in activation of the inflammatory response (NASH). However, four weeks of atherogenic diet administration significantly induced hepatic inflammation and macrophage infiltration which allowed us to evaluate fundamental pathological role of inflammation in this disease model, without the confounding effects of weight gain, adipose tissue inflammation, and insulin resistance. Follow-up studies in the diet-induced obesity and methionine/choline deficient diet models of NAFLD/NASH remain necessary. In addition, although circulating levels of anti-inflammatory EETs were significantly higher in *Ephx2*$^{-/-}$ mice compared to mice administered *t*-AUCB, suggesting a “dose response” effect with complete abrogation of sEH activity producing a greater effect than partial pharmacologic inhibition, we have not fully evaluated this hypothesis. Moreover, the inability of high-dose administration of *t*-AUCB to produce a significant anti-inflammatory effect, despite clear inhibition of sEH, suggests that in order to have utility as an anti-inflammatory therapeutic approach to prevent the

progression of fatty liver disease, pharmacologic inhibition of sEH must increase EETs to levels similar to those achieved by *Ephx2* disruption.

Conclusions

In summary, we have demonstrated that in response to an atherogenic diet hepatic CYP epoxygenase activity, and both hepatic and circulating levels of anti-inflammatory EETs are suppressed. These data suggest that suppression of hepatic CYP-mediated EET biosynthesis is an important pathological consequence of NAFLD/NASH *in vivo*. In addition, genetic disruption of sEH restored hepatic and circulating EET levels, and attenuated hepatic inflammation. Collectively, these data indicate that the CYP epoxygenase pathway is an important regulator of the NAFLD/NASH-associated hepatic inflammatory response. Future studies are needed to improve our understanding of the mechanisms underlying the role of sEH and EETs in the regulation of NAFLD/NASH-associated chronic hepatic inflammation.

Tables

Table 5.1. Hepatic inflammation scores by treatment group.

Group	0	1	2	3
Standard Diet				
WT (control)	18 (100%)	0 (0%)	0 (0%)	0 (0%)
Atherogenic Diet				
WT	12 (28%)	9 (21%)	13 (30%)	9 (21%)
Ephx2$^{-/-}$	14 (58%)	4 (17%)	5 (21%)	1 (4%)
CYP2J2-Tr	2 (13%)	5 (33%)	3 (20%)	5 (33%)
WT + *t*-AUCB	1 (8%)	3 (23%)	6 (46%)	3 (23%)
WT + HD *t*-AUCB	3 (21%)	4 (29%)	1 (7%)	6 (43%)

Data presented as the n (percentage) of mice from each group having an inflammation score of 0, 1, 2, or 3.

Note: percentages may not equal 100% due to rounding.

The overall chi-square analysis demonstrated a significant induction of inflammation ($p<0.001$).

Ephx2$^{-/-}$ mice were significantly more likely to have lower levels of inflammation ($p=0.007$), no other treatment groups were significantly different from WT mice administered the atherogenic diet using ordinal logistic regression.

Figures

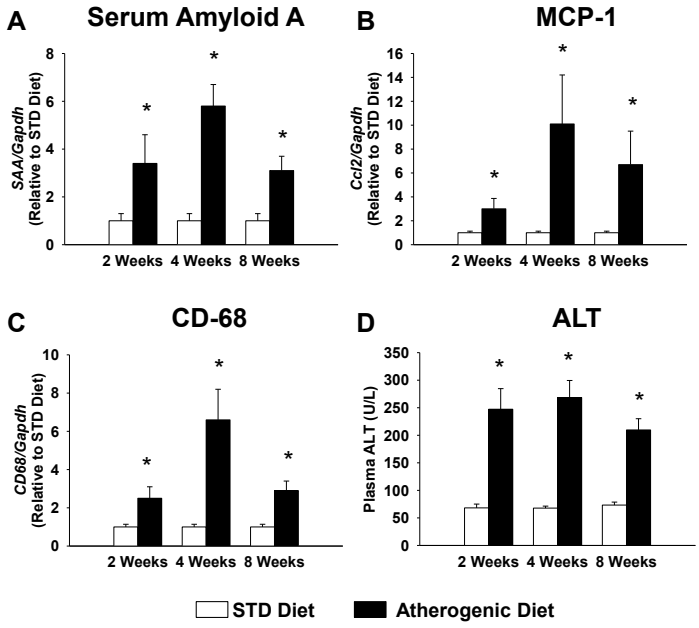

Figure 5.1. Time course of atherogenic diet-induced inflammation and liver damage. (A) Serum amyloid A, (B) MCP-1, and (C) CD-68 mRNA levels were significantly induced in mice administered the atherogenic diet for 2, 4, or 8 weeks (n=6 per group) relative to STD diet fed mice (n=15). (D) Plasma ALT was significantly increased in mice administered the atherogenic diet for 2, 4, or 8 weeks (n=6 per group) compared to mice administered the STD diet (n=15). Data presented as mean ± SEM. *P<0.05 vs. STD diet group.

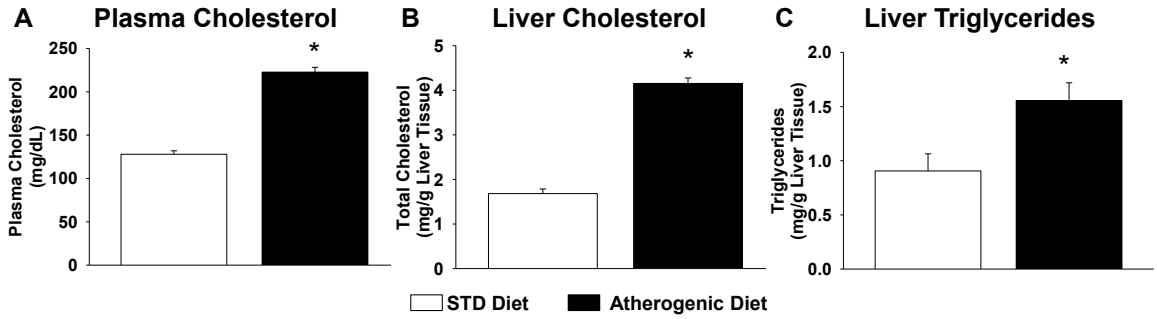

Figure 5.2. Plasma and hepatic lipid levels in response to atherogenic diet administration.

(A) Plasma total cholesterol levels, (B) liver total cholesterol levels, and (C) liver triglyceride levels were significantly higher in mice administered the atherogenic diet for 4 weeks compared to mice administered the STD diet (plasma total cholesterol STD diet: n=10, atherogenic diet: n=22; liver cholesterol STD diet: n=6, atherogenic diet: n=14; liver triglycerides STD diet: n=6, atherogenic diet n=13). Data presented as mean ± SEM. *P<0.05 vs. STD diet group.

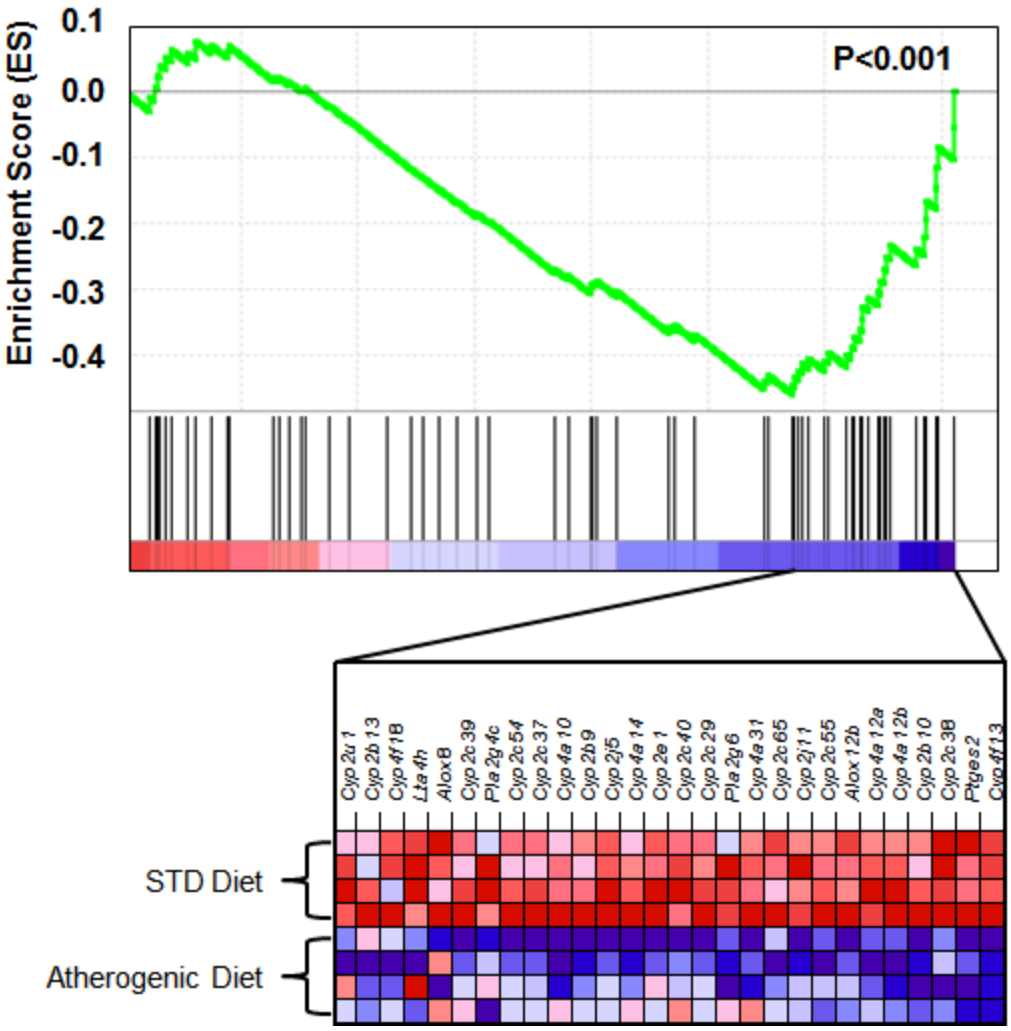

Figure 5.3. Arachidonic acid metabolism pathway gene expression in response to atherogenic diet administration.

Gene expression profiling was completed using the Agilent Whole Mouse Genome Microarray (n=4 per group). To generate the enrichment plot, each gene on the microarray is rank-ordered (left to right) according to its correlation with atherogenic diet administration (most positive on the far left, most negative on the far right). The enrichment plot for the arachidonic acid metabolism pathway GSEA indicates the position of each gene (vertical lines) within the pathway in the overall rank-order of the correlation (top of figure). A heat-map is provided illustrating gene expression levels for each gene in the core enrichment subset (bottom of figure), blue indicates the gene is down-regulated and red indicates the gene is up-regulated. The p-value for the GSEA is provided.

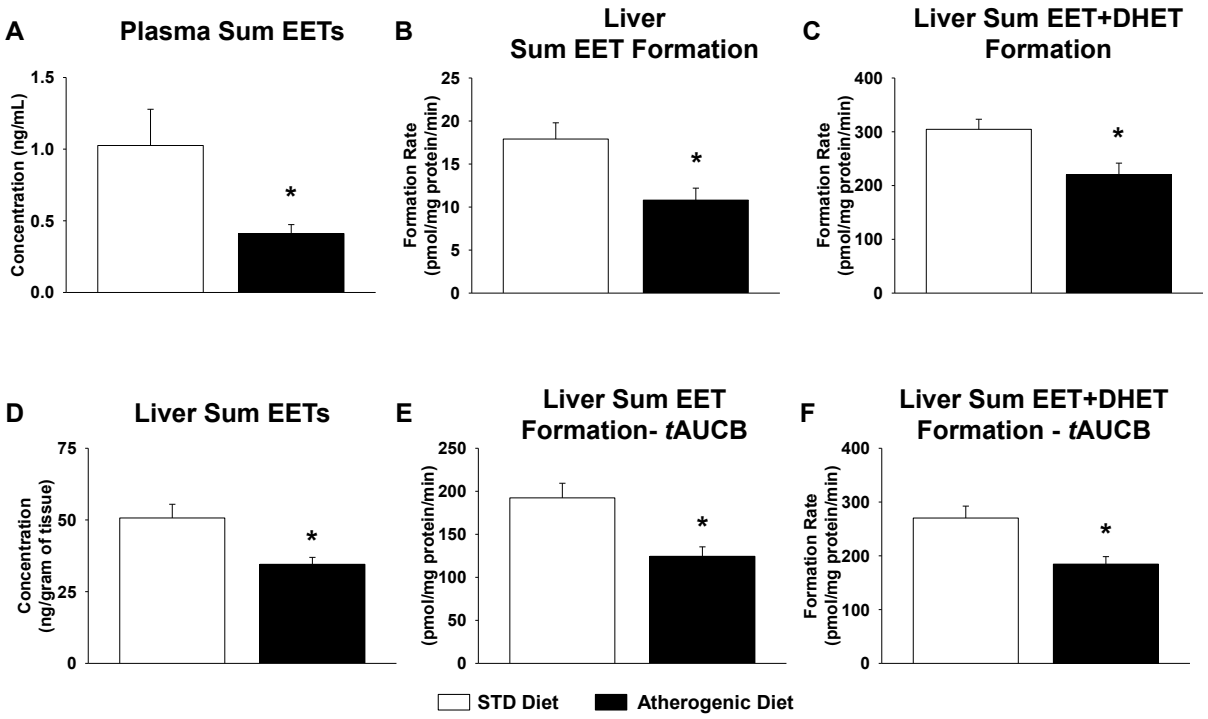

Figure 5.4. Atherogenic diet administration suppresses the CYP epoxygenase pathway.

(A) Plasma sum EET levels (STD diet: n=6, atherogenic diet: n=13) and (D) liver sum EET levels (STD diet: n=3, atherogenic diet n=5) were significantly lower in mice administered the atherogenic diet compared to mice administered the STD diet. Formation rates for (B, E) sum EETs and (C, F) sum EETs+DHETs are suppressed in liver microsomes of mice administered the atherogenic diet compared to mice administered the STD chow diet in the presence and absence of the sEH inhibitor *t*-AUCB (n=8 per group). Data presented as mean ± SEM. *P<0.05 vs. STD diet group.

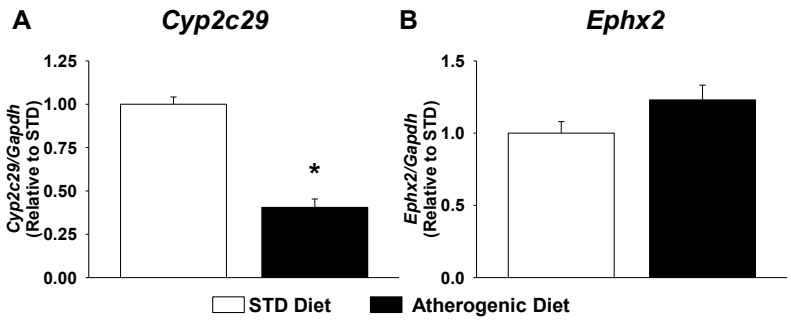

Figure 5.5. Effects of Atherogenic diet feeding on CYP epoxygenase pathway mRNA levels.

(A) Liver *Cyp2c29* mRNA levels were significantly lower but (B) *Ephx2* mRNA levels were not significantly different in mice administered the atherogenic diet compared to mice administered the STD diet (n=6 per group). Data presented as mean ± SEM. *P<0.05 vs. STD diet group.

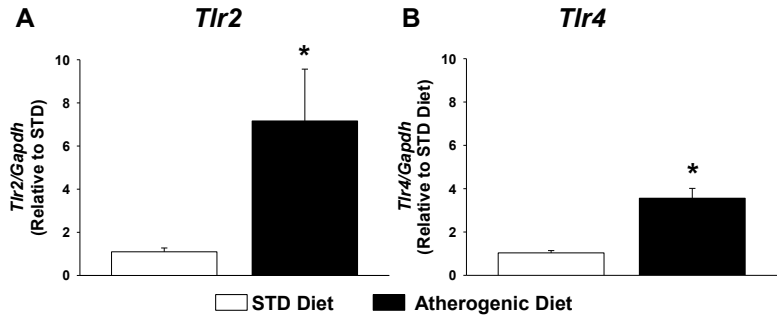

Figure 5.6. Toll like receptor expression in response to atherogenic diet administration.

Hepatic (A) *Tlr2* and (B) *Tlr4* mRNA levels were significantly induced following four weeks of atherogenic diet administration (STD diet group n=9, atherogenic diet group n=6). Data presented as mean ± SEM. *P<0.05 vs. STD diet group.

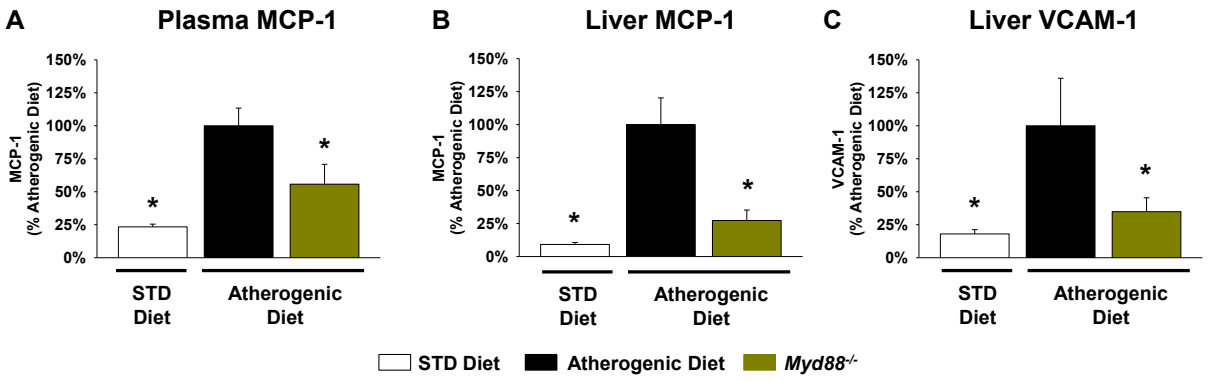

Figure 5.7. *Myd88* disruption attenuates atherogenic diet induced inflammation.
Disruption of Myd88 signaling attenuates atherogenic diet induced protein expression of (A) plasma MCP-1 (B) liver MCP-1, and (C) liver VCAM-1, (STD Diet: n=6, atherogenic diet: n=5, *Myd88*$^{-/-}$: n=5). Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group.

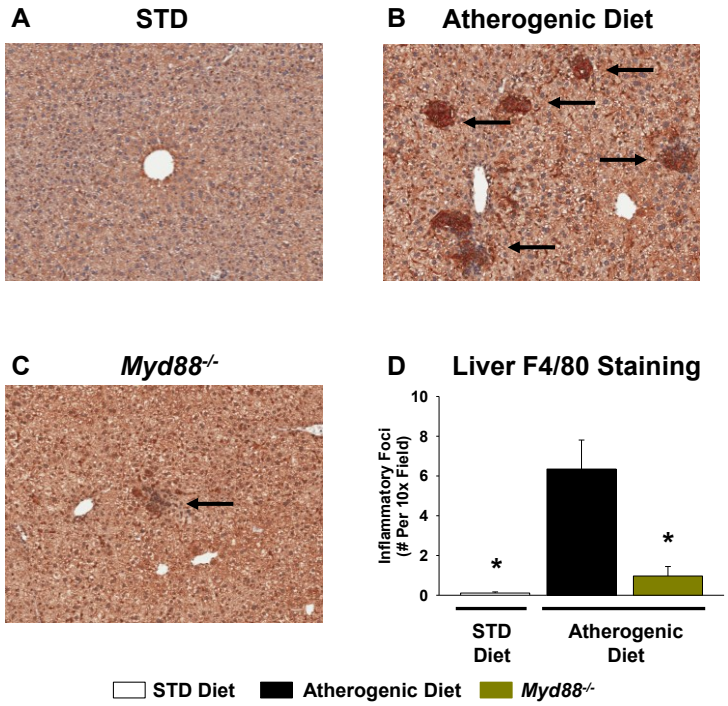

Figure 5.8. *Myd88* disruption attenuates atherogenic diet induced macrophage infiltration into hepatic tissue.

Representative images of F4/80 stained slides from (A) STD diet (n=6), (B) atherogenic diet (n=5), and (C) *Myd88*$^{-/-}$ (n=5) mice are provided. (D) The number of inflammatory foci per 10x field was significantly increased in mice administered the atherogenic diet and significantly attenuated in *Myd88*$^{-/-}$ mice. Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group.

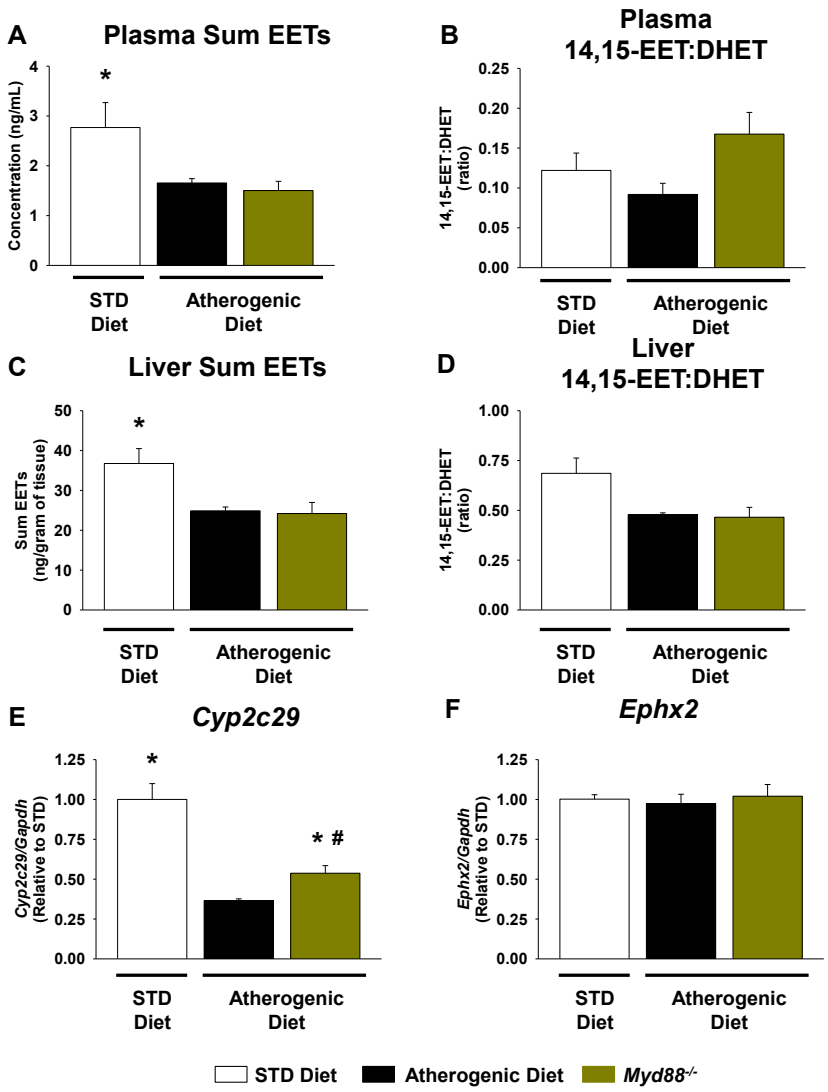

Figure 5.9. Effect of *Myd88* disruption on atherogenic diet induced changes in the CYP epoxygenase pathway.

(A) Plasma and (C) liver sum EET levels were significantly lower in WT mice administered the atherogenic diet compared to the STD diet group; however, genetic disruption of *Myd88* had no effect on sum EET levels. The 14,15-EET:DHET ratio was not significantly impacted by the atherogenic diet in (B) plasma or (D) liver tissue. (E) Hepatic *Cyp2c29* expression was significantly suppressed in mice administered the atherogenic diet, and partially restored in *Myd88*$^{-/-}$ mice. (F) No differences were observed in *Ephx2* expression (STD Diet: n=6, atherogenic diet: n=5, *Myd88*$^{-/-}$: n=5). Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group.

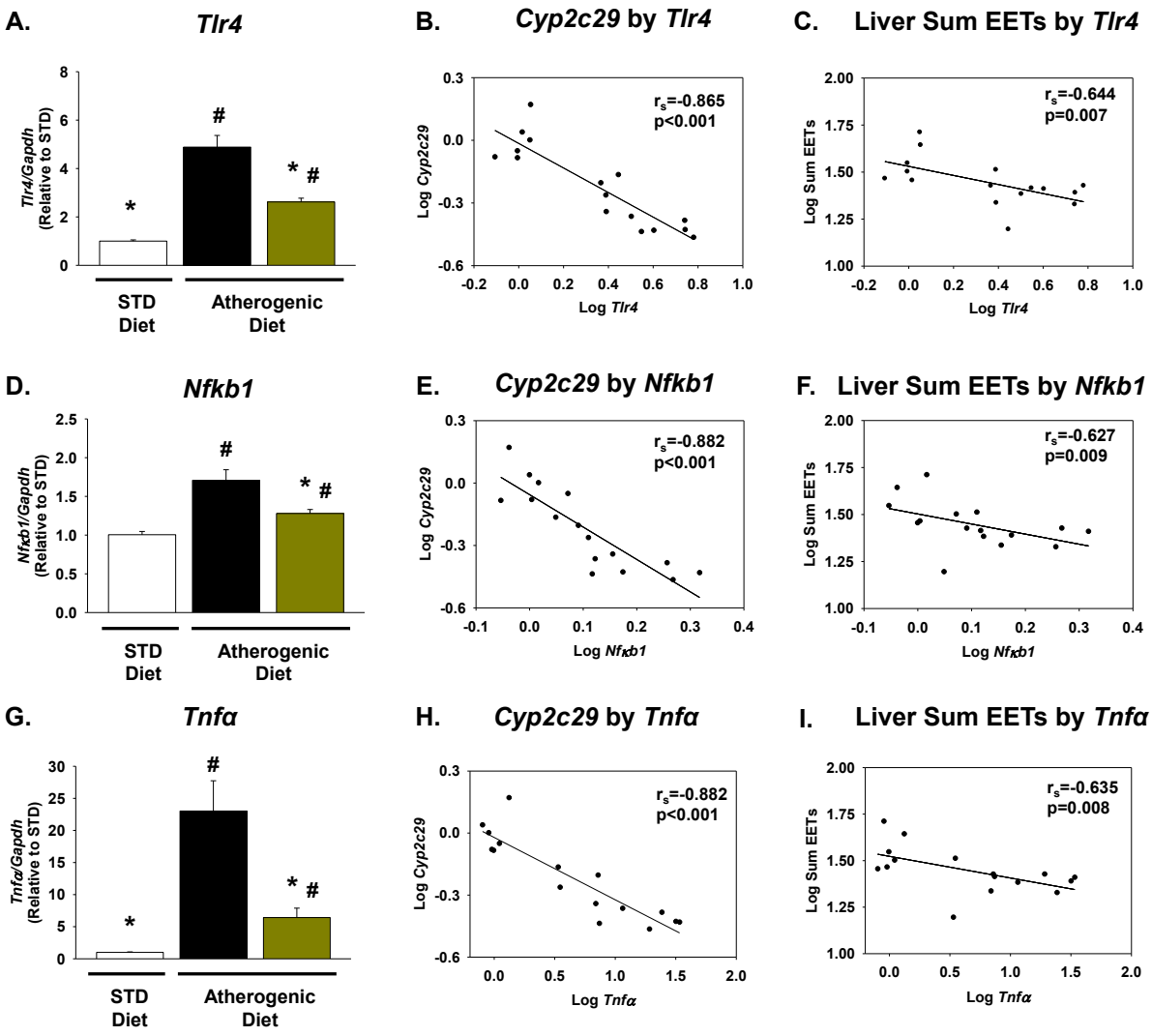

Figure 5.10. Residual inflammation and immune activation correlates with CYP epoxygenase pathway activity.

(A, D, G) Administration of the atherogenic diet increases hepatic *Tlr4, Nfκb1,* and *Tnfα* mRNA levels, and each is significantly attenuated in *Myd88*$^{-/-}$ mice but remain significantly elevated compared to STD diet fed mice. (B, E, H) Hepatic expression of *Cyp2c29* is inversely correlated with hepatic expression of *Tlr4, Nfκb1,* and *Tnfα*. (C, F, I) Liver sum EET levels are inversely correlated with hepatic expression of *Tlr4, Nfκb1,* and *Tnfα* (STD Diet: n=6, atherogenic diet: n=5, *Myd88*$^{-/-}$: n=5). Data presented as mean ± SEM, or individual data points for log-transformed gene expression and liver EET levels. *P<0.05 vs. atherogenic diet group, #P<0.05 vs. STD diet.

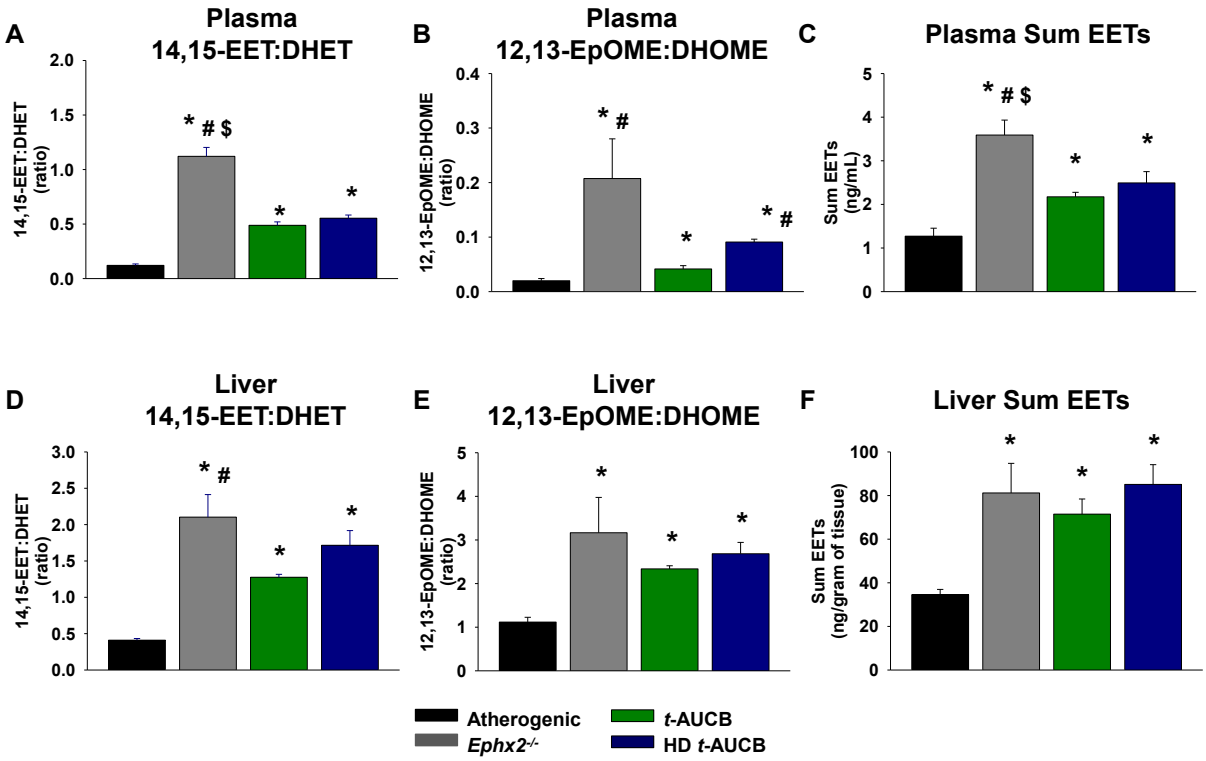

Figure 5.11. Genetic disruption and pharmacologic inhibition of sEH restores circulating and hepatic EET levels.

Plasma and liver (A,D) 14,15-EET:DHET and (B,E) 12,13-EpOME:DHOME ratios and (C,F) sum EET levels are significantly higher in *Ephx2*$^{-/-}$ mice and the standard (10 mg/L) and high-dose (50 mg/L) *t*-AUCB treated groups compared to mice administered the atherogenic diet. The plasma 14,15-EET:DHET ratio and plasma sum EETs are significantly higher in *Ephx2*$^{-/-}$ mice compared to each of the *t*-AUCB treated groups and the liver 14,15-EET:DHET ratio is significantly higher than the standard dose *t*-AUCB treated group. The plasma 12,13-EpOME:DHOME ratio was significantly higher in *Ephx2*$^{-/-}$ mice and mice administered high-dose *t*-AUCB compared to standard dose *t*-AUCB (plasma atherogenic diet: n=9, *Ephx2*$^{-/-}$, n=5, *t*-AUCB: n=6, HD *t*-AUCB: n=7; liver atherogenic diet: n=5, *Ephx2*$^{-/-}$, n=5, *t*-AUCB: n=3, HD *t*-AUCB: n=5). Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group, $^{\#}$P<0.05 vs. *t*-AUCB group, $^{\$}$P<0.05 vs. HD *t*-AUCB group.

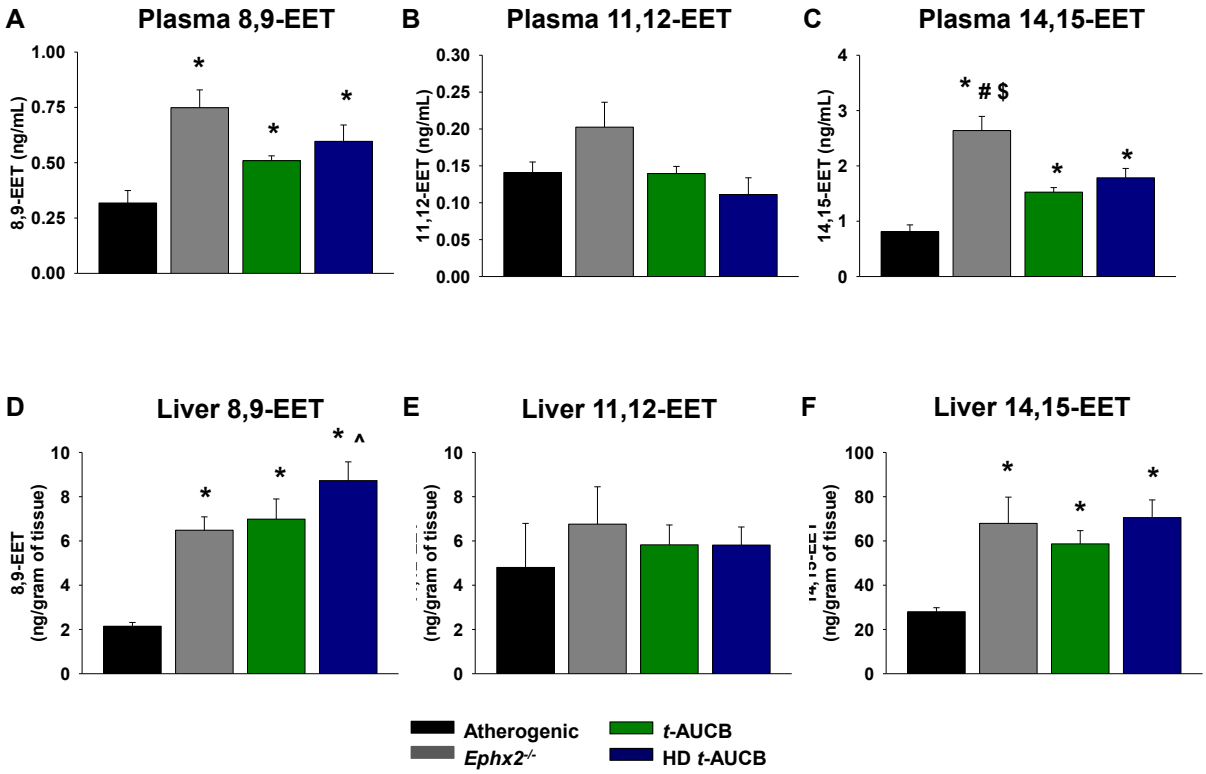

Figure 5.12. Plasma and liver concentrations of individual EET regioisomers.

(A) Plasma and (D) liver levels of 8,9-EET and were significantly higher in *Ephx2*$^{-/-}$ mice and the standard (10 mg/L) and high-dose (50 mg/L) *t*-AUCB treated groups compared to the atherogenic diet group. (B) Plasma and (E) liver 11,12-EET levels tended to be higher in *Ephx2*$^{-/-}$ mice and in each of the *t*-AUCB treated groups compared to the atherogenic diet group, but the difference did not reach statistical significance. (C) Plasma and (F) liver concentrations of 14,15-EET were significantly higher in *Ephx2*$^{-/-}$ mice and in each of the *t*-AUCB treated groups compared to the atherogenic diet group, and plasma 14,15-EET levels were significantly higher in *Ephx2*$^{-/-}$ mice compared to each of the *t*-AUCB treated groups (plasma atherogenic diet: n=9, *Ephx2*$^{-/-}$: n=5, *t*-AUCB: n=6, HD *t*-AUCB: n=7; liver atherogenic diet: n=5, *Ephx2*$^{-/-}$, n=5, *t*-AUCB: n=3, HD *t*-AUCB: n=5). Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group, $^{\#}$P<0.05 vs. *t*-AUCB group, $^{\$}$P<0.05 vs. HD *t*-AUCB group, ^P<0.05 vs. *Ephx2*$^{-/-}$ group.

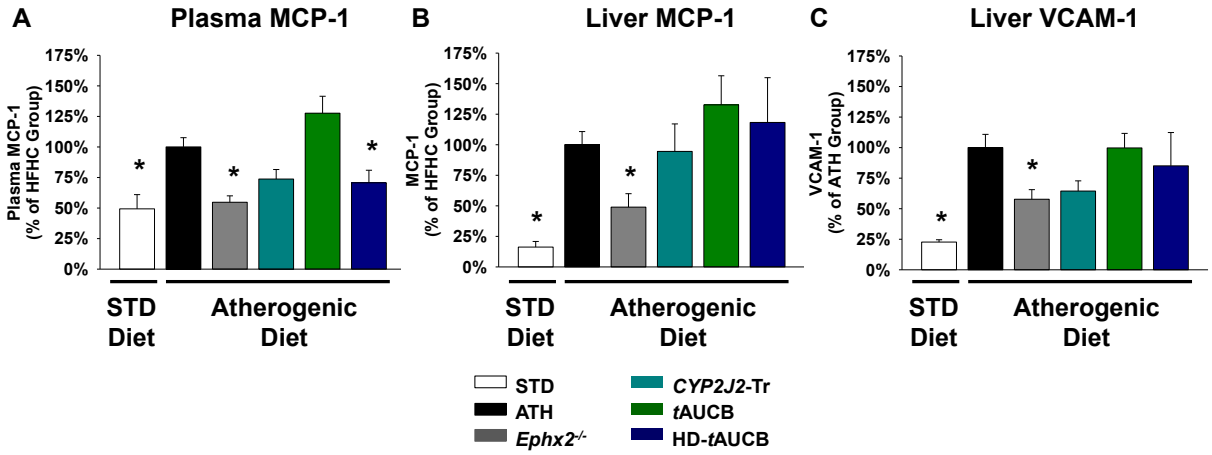

Figure 5.13. Genetic disruption of sEH attenuates hepatic expression of inflammatory biomarkers.

Plasma MCP-1, liver MCP-1, and liver VCAM-1 protein levels are significantly induced in atherogenic diet fed mice. The induction of plasma MCP-1, liver MCP-1, and liver VCAM-1 protein levels is attenuated in *Ephx2*$^{-/-}$ mice; however, no attenuation was observed in the *CYP2J2*-Tr mice or either of the *t*-AUCB treated groups (STD diet: n=15-19, atherogenic diet: n=38-45, *Ephx2*$^{-/-}$, n=22-24, C*YP2J2*-Tr: n=13-15, *t*-AUCB: n=13-14, HD *t*-AUCB: n=14). Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group.

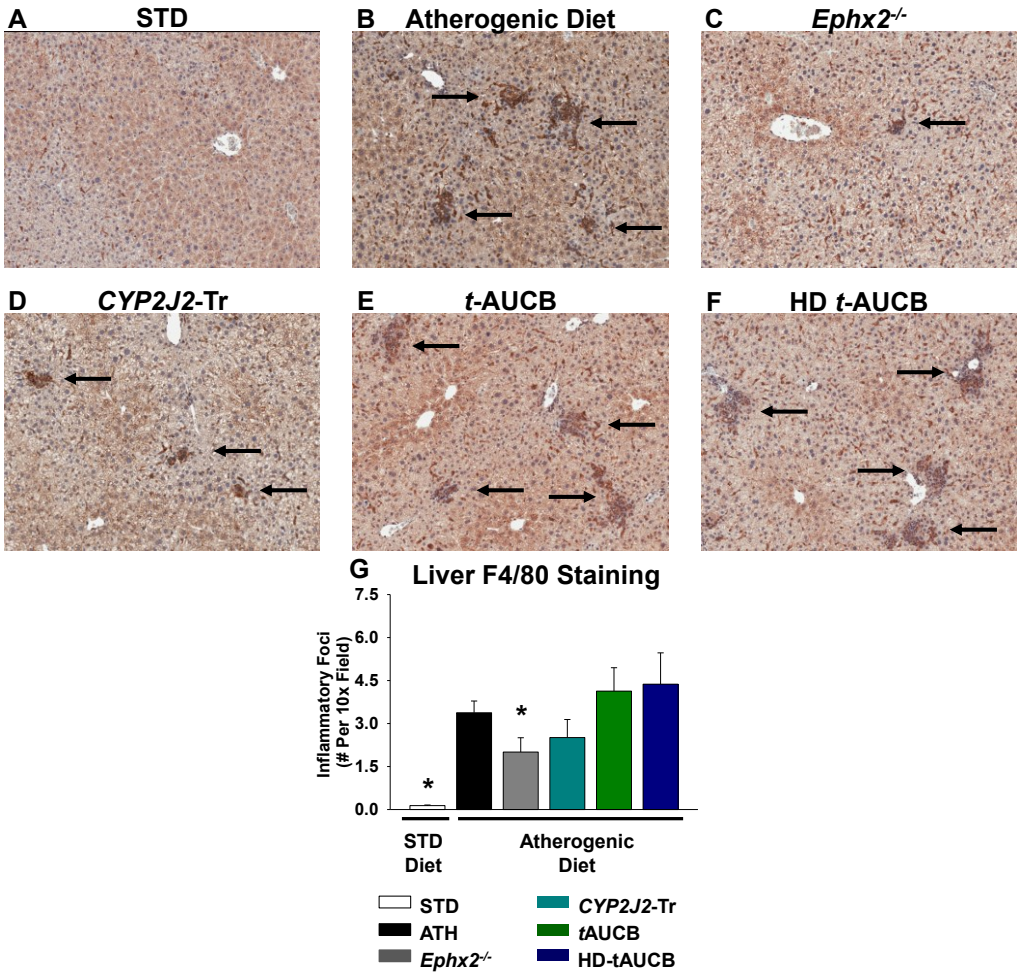

Figure 5.14. Genetic disruption of sEH attenuates macrophage infiltration into hepatic tissue.

Representative F4/80 stained images at 10x magnification from (A) STD diet (n=16), (B) atherogenic diet (n=38), (C) *Ephx2*$^{-/-}$ (n=22), (D) *CYP2J2*-Tr (n=14), *t*-AUCB (n=14), and HD *t*-AUCB (n=8) groups. (G) The number of inflammatory foci per 10x field was significantly increased in mice administered the atherogenic diet and significantly attenuated in *Ephx2*$^{-/-}$ mice, but not *CYP2J2*-Tr mice or either of the t-AUCB treated groups. Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group.

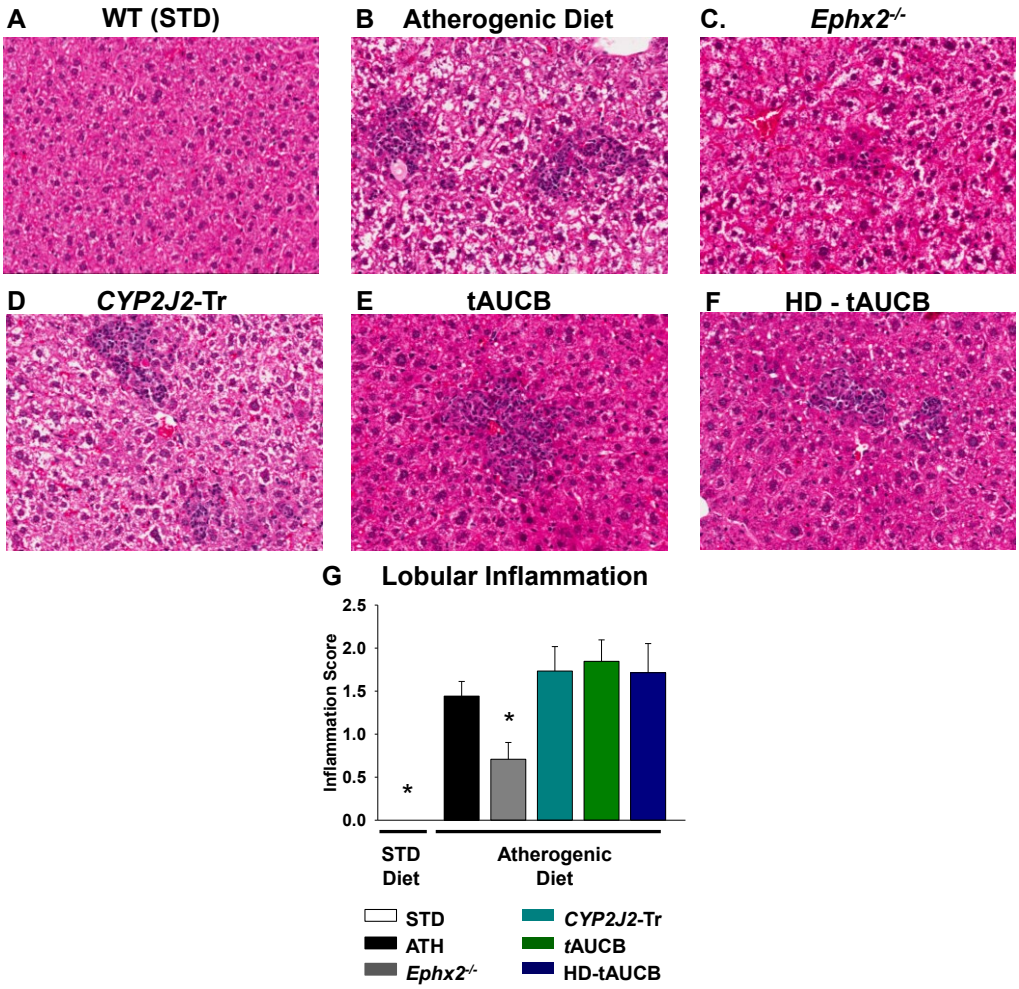

Figure 5.15. NASH lobular inflammation scoring.

Representative H&E stained images at 20x magnification from (A) STD diet (n=18), (B) atherogenic diet (n=43), (C) *Ephx2*$^{-/-}$ (n=24), (D) *CYP2J2*-Tr (n=15), (E) *t*-AUCB (n=13), and (F) HD *t*-AUCB (n=13) groups. (G) Graphical representation of the lobular inflammation score for each group demonstrates that *Ephx2*$^{-/-}$ mice have attenuated lobular inflammation score in response to atherogenic diet administration. Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group.

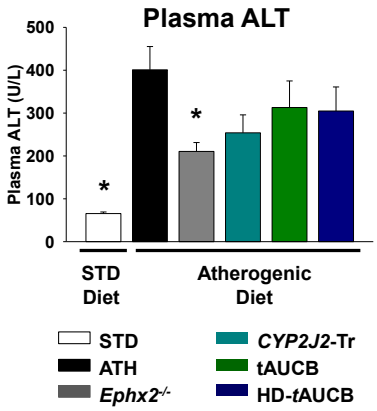

Figure 5.16. Genetic disruption of sEH attenuates induction of plasma ALT levels.

Plasma ALT levels were significantly higher in mice administered the atherogenic diet compared to mice administered the STD diet and significantly attenuated in *Ephx2*$^{-/-}$ mice, but not *CYP2J2*-Tr mice or WT mice treated with either dose of *t*-AUCB (STD diet: n=13, atherogenic diet: n=26, *Ephx2*$^{-/-}$: n=14, *CYP2J2*-Tr: n=8, *t*-AUCB: n=7, HD *t*-AUCB: n=13). Data presented as mean ± SEM. *P<0.05 vs. atherogenic diet group.

CHAPTER VI

DISCUSSION AND PERSPECTIVE

Summary and Scope

Cardiovascular disease (CVD) is a major public health problem, accounting for 1 in every 3 deaths in the United States.[1] Moreover, individuals with established coronary artery disease (CAD) are at high risk for acute coronary syndrome (ACS) clinical events, which are associated with significant morbidity and mortality. While substantial progress has been made in the treatment of CAD in recent decades, novel therapeutic approaches are needed to further improve prognosis in CAD patients. However, recent failures in drug development indicate that new therapeutics are unlikely to be effective in broadly defined populations. Therefore, thorough understanding of the mechanisms underlying the progression of CAD is necessary to facilitate the development and rigorous evaluation of novel therapeutic strategies, and identify putative responders to targeted therapies for the treatment of CAD.

Atherosclerosis is a chronic disease with complex underlying pathophysiology; however, it is well-established that vascular and systemic inflammation is integral to the development and progression of CAD, and the liver is a critical mediator of the initiation and propagation of the inflammatory response.[3, 4] Moreover, emerging evidence indicates that cytochrome P450 (CYP)-derived eicosanoids are key regulators of inflammation and endothelial function, and modulation of CYP-mediated arachidonic acid metabolism has emerged as a potential therapeutic target for the treatment of CAD. However, the functional association between CYP-mediated eicosanoid metabolism, inflammation, and CAD remains poorly

understood, and requires rigorous investigation in preclinical models and humans. Thus, the overall aim of this dissertation was to characterize the functional contribution of CYP-mediated eicosanoid metabolism to the regulation of systemic and vascular inflammation within the clinical context of CAD. We have taken a translational research approach using preclinical models and humans to evaluate the functional relationship between CYP-mediated eicosanoid metabolism, systemic and vascular inflammation, and CAD. The major findings of this work include 1) the identification of key genes associated with the presence and severity of CAD, which may represent novel targets for therapeutic strategies that aim to prevent CAD development and progression, 2) the observation of an association between dysregulation of CYP-mediated eicosanoid metabolism in human CAD patients and vascular dysfunction phenotypes that are predictive of prognosis in this patient population, and 3) the discovery that suppression of CYP epoxygenase pathway activity is a key pathological consequence of fatty liver disease-associated inflammation, an emerging risk factor for CAD, and reversal of this suppression by genetic disruption of sEH attenuates fatty liver disease-associated inflammation.

The major implication of this work includes laying a critical foundation for the rational design of future studies that seek to rigorously evaluate CYP epoxygenase pathway potentiation as an anti-inflammatory approach for the treatment of non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH), as well as CAD.

Key Findings

Systemic and vascular inflammation are key drivers of the pathogenesis and progression of CAD, and the liver is a critical mediator of the initiation and propagation of the inflammatory response.[115] Therefore, in order to characterize the contribution of systemic and vascular inflammation to the development and progression CAD in older adults, we first utilized a global systems biology approach to identify the key biological processes that

underlie the development and progression of obstructive CAD in humans (Aim 1). Our analysis in the Supporting a Multidisciplinary Approach to Researching Atherosclerosis (SAMARA) cohort is the first study to rigorously evaluate the key mechanisms underlying the pathophysiology of CAD in older adults using a combination of non-biased systems biology and candidate biomarker approaches.

Consistent with our hypothesis, and previous observations in the general population, our global gene expression analysis in peripheral blood mononuclear cells (PBMCs) confirmed that inflammation is an important pathological mediator underlying the pathogenesis and progression of CAD in older adults. We identified multiple genes that may be important in the development and progression of CAD in older individuals, including *CASP4*, *HLA-E*, and *MAPK14,* which were associated with the presence and severity of CAD in previous studies in younger patient populations.[104] Since the association between expression of these genes and the presence and severity of CAD has now been validated in multiple independent patient populations, future studies using preclinical models of atherosclerosis are warranted to directly evaluate their functional role in the pathogenesis and progression of atherosclerotic CAD. Additional genes identified in our analysis were not found to be associated with the presence and severity of CAD in previous studies with younger patient populations, and therefore may be unique to the pathophysiology of CAD in older adults; however, validation of these findings in an independent cohort remains necessary. Future studies directly evaluating the unique mechanisms driving the development and progression of CAD in older adults are warranted.

Our candidate biomarker analysis demonstrated that elevated circulating biomarkers of inflammation, which are established biomarkers of CAD risk in the general population, are not predictive of CAD status in older adults. These data suggest that circulating biomarkers of inflammation have limited clinical utility for risk assessment in this population and more advanced biomarker analyses, such as global gene expression profiling in PBMCs, may be

necessary to improve the precision with which we assess risk in older adults. Future studies evaluating the role of novel circulating biomarkers of CAD risk in older adults remain necessary. Collectively, our candidate biomarker and global gene expression analyses suggest that the role of inflammation in the pathophysiology of CAD in the elderly is not captured by circulating biomarkers, and the mechanism by which inflammation impacts the development and progression of CAD in older individuals may be distinct from that in the general population. Importantly, inflammation was identified as a key pathological mediator of CAD in older adults, indicating that anti-inflammatory therapeutics that are currently in development for the treatment of CAD, including those that modulate CYP-mediated arachidonic acid metabolism, may have clinical utility in this patient population.

In order to characterize the contribution of CYP-mediated arachidonic acid metabolism to CAD development and progression, we took a candidate pathway approach using gene set enrichment analysis (GSEA) in the SAMARA cohort. Our analysis did not demonstrate a significant relationship between expression of the arachidonic acid metabolism pathway as a whole and CAD in PBMCs of older individuals. However, we observed a signal suggesting that expression of individual genes within the arachidonic acid metabolism pathway, most notably *EPHX2* (a key gene in the CYP pathway of arachidonic acid metabolism), may be suppressed in CAD patients. Suppression of *EPHX2*, which metabolizes epoxyeicosatrienoic acids (EETs) to the less biologically active dihydroxyeicosatrienoic acids (DHETs), in CVD patients is consistent with our previous report that sEH metabolic function is suppressed in stable CAD patients under 65 years of age compared to healthy volunteers,[136] and studies demonstrating that *EPHX2* is suppressed in individuals with ischemic heart failure compared to control subjects.[137] These findings suggest that the presence of CVD may lead to a compensatory suppression of sEH-mediated EET hydrolysis in humans that was not captured by our global analysis of arachidonic acid pathway gene expression in PBMCs, and demonstrate the need for studies evaluating the association

between inter-individual variation in CYP-derived eicosanoids at the metabolite level and the pathogenesis and progression of CAD.

Our analysis of arachidonic acid metabolism pathway gene expression in PBMCs from older adults demonstrated that genes from the CYP epoxygenase pathway may be important in the development and progression of CAD. Therefore, we utilized a candidate pathway approach to characterize the functional relationship between inter-individual variation in CYP-mediated eicosanoid metabolism and endothelial dysfunction, vascular inflammation, and systemic inflammation in stable obstructive CAD patients (Aim 2). We accomplished this by quantifying inter-individual variation in CYP-derived eicosanoids at the metabolite level, and biomarkers of vascular function that are predictive of prognosis in CAD patients. Our analysis of CYP metabolites in humans identified a subset of individuals with established CAD that have dysregulated CYP-mediated eicosanoid metabolism and vascular dysfunction, suggesting that inter-individual variation in CYP-derived eicosanoids is an important regulator of phenotypes that are associated with prognosis in this population. This is the first study in humans to demonstrate that dysregulation of CYP-mediated eicosanoid metabolism is associated with vascular dysfunction in this high-risk population. Importantly, these associations were identified in patients treated with standard of care medications, suggesting that treatment of CAD patients who have enhanced CYP ω-hydroxylase or sEH metabolic activity with adjunct therapies that inhibit 20-HETE biosynthesis and/or EET hydrolysis may improve vascular function and subsequently improve prognosis. Future studies remain necessary to validate these findings and evaluate the association between CYP ω-hydroxylase and sEH metabolic activity and clinical outcomes in CAD patients. In addition, proof-of-concept studies are necessary to define the vascular protective effects and safety of decreasing 20-HETE biosynthesis and/or inhibiting sEH activity in CAD patients.

The association between inter-individual variation in CYP-derived eicosanoids and vascular dysfunction in CAD patients suggests that targeted therapies that increase EETs and/or decrease 20-HETE may have therapeutic utility in a subset of CAD patients with dysregulated CYP-mediated eicosanoid metabolism. Importantly, pharmacologic inhibitors of sEH[139] and CYP ω-hydroxylase metabolism[140] are currently in development. Although these novel therapies are hypothesized to improve prognosis in CAD patients by eliciting protective effects in the vasculature via increasing EET and decreasing 20-HETE levels, respectively, preclinical studies are necessary to directly define the contribution of CYP-mediated eicosanoid metabolism to vascular and systemic inflammation. Therefore, we utilized a preclinical approach to define the contribution of CYP-mediated eicosanoid metabolism to the regulation of non-alcoholic fatty liver disease-associated systemic and vascular inflammation in mice (Aim 3), a preclinical model of chronic inflammation which is relevant to human CAD.

Similar to the association between dysregulated CYP-mediated eicosanoid metabolism and vascular dysfunction that was observed in CAD patients, our studies demonstrated that induction of NAFLD/NASH-associated systemic and vascular inflammation resulted in suppression of CYP epoxygenase enzymes and EET biosynthesis in the liver. Importantly, direct quantification of CYP-derived eicosanoids demonstrated that plasma and hepatic EET levels are suppressed in response to induction of NAFLD/NASH, and are highly correlated with each other, suggesting that dysregulation of CYP-mediated eicosanoid metabolism in the liver regulates circulating metabolite levels. These findings highlight the potential importance of hepatic eicosanoid metabolism in the observed relationship between circulating eicosanoid levels and vascular dysfunction in human CAD patients, and the need to directly evaluate the contribution of CYP-mediated eicosanoid metabolism to the regulation of systemic and vascular inflammation in preclinical models of sustained hepatic inflammation. Interestingly, dysregulation of the CYP epoxygenase pathway was secondary

to suppression of CYP enzymes in our preclinical model of fatty liver disease-associated inflammation, while enhanced sEH metabolic function was the key mediator of CYP epoxygenase pathway dysfunction in our analysis of human CAD patients. These findings suggest that different disease pathologies may impact CYP-mediated eicosanoid metabolism at distinct levels of the pathway, and highlight the need to rigorously characterize the mechanisms underlying dysregulation of CYP-mediated eicosanoid metabolism.

We investigated the role of innate immune system activation, a pathological process key in the development and progression of both NAFLD/NASH and CAD, [156] in the suppression of the CYP epoxygenase pathway following induction of a sustained inflammatory response. Our studies demonstrated that the innate immune system was activated in the atherogenic diet model of NAFLD/NASH; however, abolishing signaling through myeloid differentiation factor 88 (Myd88) did not restore CYP-mediated eicosanoid metabolism. Interestingly, *Tlr4, Nfkb1,* and *Tnfα* expression remained significantly elevated in *Myd88*$^{-/-}$ mice and each were inversely correlated with CYP epoxygenase expression and liver EET levels. These findings indicate that Myd88-indpendent activation of the innate immune system may have a significant role in the suppression of CYP epoxygenase activity; alternatively, Myd88-indpendent pathways may play an important “reserve” role in the absence of Myd88 signaling. Follow-up experiments remain necessary to directly evaluate the role of Myd88-indpendent activation of the innate immune system on suppression of the CYP epoxygenase pathway in fatty liver disease-associated inflammation.

Evaluation of the therapeutic effects of CYP epoxygenase pathway potentiation in fatty liver disease-associated inflammation demonstrated that genetic disruption of sEH elicits potent anti-inflammatory effects in a preclinical model of inflammation relevant to CAD. Of note, genetic disruption of sEH exhibited anti-inflammatory effects in this model despite the observation that atherogenic diet administration suppressed CYP epoxygenase expression

but had no effect on *Ephx2* expression. This is the first *in vivo* study to demonstrate that increasing circulating and hepatic EET levels may have utility as an anti-inflammatory therapeutic strategy for the treatment of NAFLD/NASH, and suggests that sEH is a viable therapeutic target, even when dysregulation of the CYP epoxygenase pathway is not secondary to changes in sEH activity or expression. Our analysis adds to a growing body of literature in the eicosanoid field demonstrating that modulation of CYP-mediated arachidonic acid metabolism is a rational anti-inflammatory therapeutic strategy for the treatment of inflammatory diseases. However, it must be noted that pharmacologic inhibition of sEH and endothelial over-expression of CYP2J2, did not produce similar anti-inflammatory effects. Future studies remain necessary to determine the mechanism underlying the observed absence of an anti-inflammatory effect in mice treated with a pharmacologic inhibitor of sEH. Importantly, more potent inhibitors of sEH are currently in development that will facilitate these investigations.[183]

Clinical Implications

In recent decades considerable progress has been made in the development of therapeutics for the treatment of CAD; however, the burden of CAD remains high and the associated morbidity and mortality highlights the need for new therapies that target biological pathways integral to its pathogenesis and progression. Recent studies evaluating adjunct therapies that lower LDL cholesterol, a well-established pathological mediator of CAD, have failed to demonstrate an improvement in clinical outcomes in broadly defined CAD patient populations (Appendix II). These disappointing results demonstrate a critical need for novel adjunct therapies targeting biological pathways that are dysregulated in CAD patients, and identifying a subset of putative responders to these therapies.

Our study identified a subset of CAD patients that have dysregulated CYP-mediated eicosanoid metabolism, and demonstrated that this subset of CAD patients exhibited

vascular dysfunction, which is predictive of poor outcomes. These findings suggest that CAD patients with dysregulated eicosanoid metabolism may represent a subset of putative responders to adjunct therapies that inhibit EET hydrolysis and/or 20-HETE biosynthesis. Moreover, in a mouse model of hepatic inflammation that precedes the development of atherosclerosis, we demonstrated that CYP epoxygenase-mediated EET biosynthesis was suppressed, and restoring EET levels by disrupting sEH-mediated EET hydrolysis resulted in reduced systemic and hepatic inflammation and liver injury. Collectively, these studies suggest that therapeutic interventions that restore CYP epoxygenase pathway metabolic function may be a viable anti-inflammatory therapeutic strategy in pathological conditions that suppress EET levels, and this therapeutic approach requires more rigorous investigation. This dissertation work has laid the foundation for future studies in this area that will focus on the critical evaluation of pharmacologic agents currently in development that increase EETs (sEH inhibitors), promote the effects of EETs (stable EET analogs), or decrease 20-HETE (CYP ω-hydroxylase inhibitors) as targeted anti-inflammatory therapeutic strategies for the treatment of CAD patients with enhanced sEH and/or CYP ω-hydroxylase metabolic function. This personalized medicine approach, which incorporates rigorous characterization of therapeutic strategies in preclinical models and identification of putative responders in humans, offers enormous potential to develop targeted therapeutic strategies with high potential to improve clinical outcomes.

Conclusions

In summary, the overall aim of this dissertation was to characterize the functional contribution of CYP-mediated eicosanoid metabolism to the regulation of systemic and vascular inflammation within the clinical context of CAD. Our functional analysis of genes associated with the presence and severity of obstructive CAD reflected differential expression of genes regulating the inflammatory response. In addition, we identified a

subset of stable obstructive CAD patients with enhanced CYP ω-hydroxylase and sEH metabolic function who had advanced endothelial dysfunction and vascular inflammation despite receiving current standard of care therapies. Moreover, our preclinical studies directly demonstrated that fatty liver disease-associated inflammation suppressed hepatic CYP epoxygenase activity, and genetic disruption of sEH restored hepatic and circulating EET levels, and attenuated hepatic and systemic inflammation. Collectively, this dissertation demonstrates that targeted therapeutic strategies that modulate CYP-mediated arachidonic acid metabolism represents a rational anti-inflammatory approach for the treatment of CAD, and thus lays a critical foundation for future studies that directly evaluate the therapeutic effects of modulating CYP-mediated eicosanoid metabolism in CAD patients.

APPENDIX I

DEVELOPMENT OF AN INFLAMMATION SCORE AS A COMPREHENSIVE PHENOTYPIC INDEX OF INFLAMMATION

Introduction

It is well-established that systemic and vascular inflammation are important in the development and progression of coronary artery disease (CAD) in the general population;[1, 2] however, studies evaluating the association between established biomarkers of inflammation and prognosis in older patient populations have not consistently demonstrated that elevated biomarkers of inflammation are associated with poorer outcomes.[3, 4] Moreover, our analysis of candidate circulating biomarkers of inflammation, which are critical mediators of systemic and vascular inflammation did not demonstrate that individual biomarkers of inflammation were significantly higher in obstructive CAD patients in the SAMARA cohort compared to individuals without obstructive CAD (Chapter II). However, analysis of individual biomarkers of inflammation captures only isolated components of the inflammatory response (i.e. acute phase response, chemoattraction, or adhesion of leukocytes to the vessel wall), whereas the pathologic inflammatory response observed in cardiovascular disease encompasses all of these processes. Thus, it has been proposed that a combined phenotypic index of inflammation or "inflammation score" may provide further insight into the relationship between inflammation and cardiovascular disease.[5] Therefore, in order to more rigorously assess the relationship between systemic and vascular inflammation and CAD in older individuals, we 1) developed a comprehensive inflammation score that captures the

inflammatory response at multiple levels and 2) characterized the association between the inflammation score and the presence and severity of obstructive CAD in older adults.

Methods

Development of an Inflammation Score

In an independent cohort of 95 stable CAD patients and 42 healthy volunteers with no cardiovascular risk factors,[6, 7] we developed a phenotypic index of inflammation "inflammation score". In fresh serum, high-sensitivity C-reactive protein (hs-CRP) was quantified by latex-enhanced turbidimetric immunoassay using the VITROS® 5600 Chemistry System (Ortho-Clinical Diagnostics, Inc., Rochester, NY). Chemokines (monocyte chemoattractant protein-1 [MCP-1] and epithelial neutrophil activating protein-78 [ENA-78]) and CAMs (intracellular adhesion molecule-1 [ICAM-1], vascular cellular adhesion molecule-1 [VCAM-1], P-selectin and E-selectin) were quantified using a multianalyte profiling kit (R&D Systems, Minneapolis, MN).

Stepwise multi-variable logistic regression was utilized to determine which biomarkers of inflammation independently differentiate a population of patients with advanced atherosclerotic cardiovascular disease from healthy volunteers at low risk for cardiovascular disease. Thus, this analysis facilitated the development of a phenotypic index of inflammation that captures the inflammatory response at multiple levels. In a univariate analysis, circulating concentrations of hs-CRP, MCP-1, ICAM-1 and E-selectin were all significantly higher in patients compared to healthy volunteers. ICAM-1 was highly correlated with E-selectin (r_s=0.57, P<0.01) and modestly correlated with hs-CRP (r_s=0.24, P<0.01). All other biomarkers included in the model were not significantly correlated with each other. In a multi-variable logistic regression model, hs-CRP (odds ratio 1.49, P=0.03), MCP-1 (odds ratio 4.28, P=0.06), and ICAM-1 (odds ratio 8.09, P<0.01) remained predictors of CAD status at the pre-defined α of 0.10. Accordingly, an inflammation score was created

in which subjects were assigned one point for each elevated (> median) biomarker of inflammation that was a significant predictor of CAD status (hs-CRP, MCP-1, and ICAM-1). The inflammation score was subsequently found to be significantly higher in subjects with CAD in this cohort of patients with established stable CAD and healthy volunteers with no cardiovascular risk factors (Figure 1), suggesting it represents biological mediators of inflammation that are key in the pathology of CAD.

This data reduction process allows us to have an index of inflammation that captures the inflammatory response at multiple levels, and thus is a more complete phenotypic index of inflammation than individual biomarkers. Consequently, this inflammation score was used as a comprehensive phenotypic index of inflammation to characterize the association between advanced inflammation and the presence and severity of obstructive CAD in the SAMARA cohort.

Quantification of Circulating Biomarkers of Inflammation

Plasma concentrations of hs-CRP, ICAM-1 and MCP-1 were quantified, and the inflammation score was calculated in 143 participants in the SAMARA cohort. ICAM-1 and MCP-1 were quantified using the Human Adhesion Molecule and Human Cytokine Fluorokine® Multi-Analyte Profiling Kits (R&D Systems, Minneapolis, MN), respectively. hs-CRP was quantified using the Human C-Reactive Protein Fluorokine® MAP kit for use in Cardiac Panel B. All analytes were quantified with fluorescence detection on the Bio-Plex 200 System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The inflammation score was calculated as described above, assigning subjects one point for each elevated (> median) biomarker of inflammation (hs-CRP, MCP-1, and ICAM-1).

CAD Severity

Subjects underwent diagnostic left-heart catheterization as part of their routine medical care. Following the procedure, CAD severity was quantified in each subject by coronary angiography by calculating a CAD score as follows: 0 (<10% stenosis in all major coronary arteries); 1 (10-70% stenosis in at least one vessel); 2 (>70% stenosis in one vessel); 3 (>70% stenosis in two vessels); and, 4 (>70% stenosis in 3 vessels or >70% stenosis in the left-main coronary artery).

Obstructive CAD was defined as >70% stenosis in ≥1 major epicardial coronary artery (CAD score 2-4). Subjects with ≤70% occlusion in any vessel (CAD score 0-1) were considered free of obstructive CAD and thus were utilized as the comparator group.

Statistical Analysis

The association between the inflammation score and CAD score was evaluated using an unadjusted regression model and a model that adjusted for potential demographic (age, race, gender) and clinical (obesity, diabetes mellitus, hyperlipidemia, statin use) confounders that associated with biomarkers of inflammation or obstructive CAD status in the SAMARA cohort. Similarly, the inflammation score was compared in subjects with and without obstructive CAD using both the unadjusted and adjusted regression models. A secondary analysis compared the proportion of subjects with an inflammation score of 0, 1, 2, or 3 in individuals with and without obstructive CAD using a Chi-square analysis.

Results

The inflammation score was not significantly associated with CAD score or the presence of obstructive CAD in this population of older individuals in either the unadjusted or adjusted model (Table 1). Similarly, the proportion of individuals with an inflammation score of 0, 1, 2,

or 3, was not significantly different in individuals with obstructive CAD compared to individuals without obstructive CAD (p=0.236, Figure 2).

Discussion

Systemic and vascular inflammation are important in the development and progression of coronary artery disease (CAD) in the general population.[1, 2] However, our analysis of candidate circulating inflammatory biomarkers in older adults did not demonstrate a significant association between higher levels of circulating inflammatory biomarkers and the presence and severity of obstructive CAD (Chapter II). Therefore we developed a comprehensive phenotypic index of inflammation "inflammation score" in order to more rigorously evaluate the association between systemic and vascular inflammation and obstructive CAD in older adults. The inflammation score was a significant predictor of CAD status in a younger population of stable, angiographically confirmed CAD patients compared to healthy volunteers with no CAD risk factors. However, this comprehensive phenotypic index of inflammation was not a significant predictor of the presence or severity of obstructive CAD in the SAMARA study, a more heterogeneous population of older individuals. These findings suggest that the inflammation score has little clinical utility for assessment of the presence of cardiovascular disease, or disease progression, in this patient population.

Conclusions

Our analysis in older adults did not demonstrate a significant association between a comprehensive inflammation score that captures the inflammatory response at multiple levels and the presence and severity of obstructive CAD. These findings highlight the need for more advanced and unbiased methods to identify key processes underlying the development and progression of obstructive CAD in older adults.

Tables

Table 1. Association between CAD score and obstructive CAD status and the inflammation score.

Analyte	Parameter Estimate	Standard Error	Partial R^2	P
CAD Score				
Unadjusted	0.057	0.066	0.005	0.388
Adjusted	0.075	0.074	0.008	0.312
Obstructive CAD				
Unadjusted	0.168	0.190	0.006	0.376
Adjusted	0.253	0.208	0.001	0.225

Adjusted model: adjusted for age, race, gender, obesity, diabetes mellitus, hyperlipidemia, statin use.

Figures

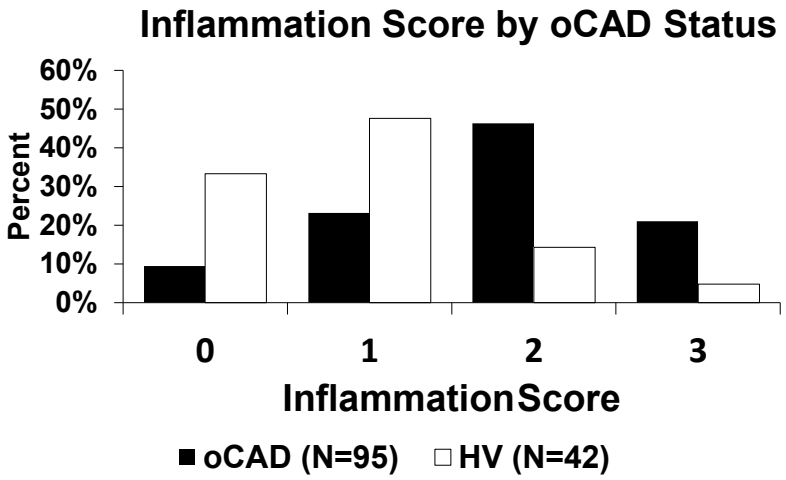

Figure 1. Development of a multivariate inflammation score that is predictive of CAD status.

The percentage of individuals with stable obstructive CAD (oCAD, black bars) and healthy volunteers (HV, white bars) having an inflammation score of 0, 1, 2, or 3. CAD is significantly higher in subjects with higher inflammation scores (Chi-Square $P<0.0001$).

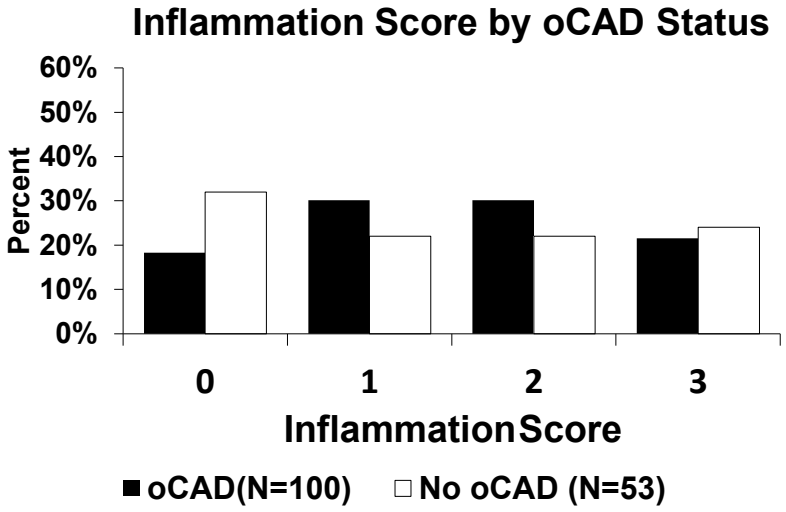

Figure 2. Inflammation Score by CAD status in the SAMARA cohort.

The percentage of individuals with obstructive CAD (oCAD, black bars) and individuals without an obstructive CAD diagnosis (No oCAD, white bars) having an inflammation score of 0, 1, 2, or 3 is displayed (Chi-Square p=0.236).

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APPENDIX II

BEYOND STATINS: LIPID MANAGEMENT TO REDUCE CARDIOVASCULAR RISK[2]

Abstract

The discovery that elevated total cholesterol levels and the subsequent understanding that low-density lipoprotein cholesterol levels are associated with higher risk for cardiovascular disease (CVD) has led to the development of lipid management strategies that seek to reduce the burden of CVD. Whereas substantive progress has been made in reducing death and cardiovascular events, questions remain regarding the optimal approach to further reduce CVD-associated death and disability. Based on current evidence, statins are the clear first-line agents for the management of hyperlipidemia in patients at high risk for cardiovascular events. However, due to the failure of recent clinical trials evaluating antihyperlipidemic drugs, the most appropriate lipid management strategy in patients who cannot tolerate statin medications or who warrant antihyperlipidemic therapies in addition to statins is a major therapeutic controversy. In this review, we summarize the clinical trial evidence evaluating the efficacy of second-line antihyperlipidemic drug classes for reducing cardiovascular risk, provide recommendations for appropriate use of nonstatin lipid-altering drugs, and identify key areas of future research to support evidence-based lipid management. Given the complexity, magnitude, and burden of CVD, opportunities to improve processes of care and identify new therapeutic options clearly exist.

[2] Schuck RN, Mendys PM, Simpson RJ Jr., Pharmacotherapy, 2013.

Introduction

Since the discovery that elevated total cholesterol levels and the subsequent understanding that low-density lipoprotein (LDL) cholesterol levels are associated with higher risk for cardiovascular disease (CVD), scientists and clinicians have worked to develop a lipid management strategy to reduce the burden of CVD using a range of epidemiological, basic science, and clinical studies. Although we have made substantial progress in reducing death and cardiovascular events, questions remain regarding the optimal approach to further reduce death and disability associated with CVD, which is the leading cause of morbidity and mortality in the United States.[1] It is well established that elevated circulating levels of LDL cholesterol are associated with higher risk of developing CVD and poor clinical outcomes in patients with established coronary disease.[2] Furthermore, numerous clinical trials have demonstrated that lowering LDL cholesterol improves clinical outcomes.

The evidence generated in clinical trials of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) convincingly supports their efficacy to improve clinical outcomes in patients at high risk for cardiovascular events according to National Cholesterol Education Program Adult Treatment Panel (ATP) III guidelines.[3] The initial statin trials demonstrated that statins reduce cardiovascular events compared to placebo in primary and secondary prevention populations with elevated LDL cholesterol levels.[4-6] Subsequently, trials showed that high-dose statin therapy is associated with greater benefit than low-dose statin therapy,[7, 8] and more recent trials have demonstrated that statins are beneficial even in the range of LDL cholesterol levels that were not previously considered elevated.[9] In addition, statins have been shown to reduce the risk of stroke after an acute coronary syndrome or stroke or transient ischemic attack.[10, 11] Although the cost-effectiveness of statins in low-risk populations has been questioned,[12] based on numerous clinical trials demonstrating their ability to reduce cardiovascular events, statins have become the clear

first-line antihyperlipidemic agents for prevention of adverse outcomes both in primary and secondary prevention populations. In addition, the availability of inexpensive generic formulations of potent statins in recent years has made statin use cost-effective in patients with a 10-year risk of coronary heart disease (CHD) as low as 5%[13]; however, the most appropriate second-line class of agents remains highly controversial.

The association between LDL cholesterol and coronary artery disease (CAD) has been established in epidemiological and genetic studies as well as clinical trials, and LDL cholesterol is integral to the pathophysiology of atherosclerosis.[14] Therefore, the ATP III guidelines issued by the National Cholesterol Education Program support the practice of lowering LDL cholesterol to goal levels based on patient risk for cardiovascular events.[15, 16] However, the clinical trials from which these goals were ascertained evaluated fixed-dose statin therapy rather than dose titration to achieve target levels. Although the efficacy and safety of treating patients to target LDL cholesterol levels has not been directly evaluated, treatment with fixed-dose statin therapy has reduced cardiovascular risk in multiple trials.[17] This has led some experts to conclude that LDL cholesterol goals are not evidence based and should not be used to guide clinical decision making.[18] New guidelines supported by the National Heart, Lung, and Blood Institute (which supports the development of the ATP guidelines) will be more narrowly focused and rely solely on recommendations from detailed systematic reviews of the available evidence.[19] Thus, the upcoming ATP IV guidelines will focus heavily on evidence generated from randomized controlled clinical trials. However, new guidelines will likely allow for clinical judgment in lipid management, meaning nonstatin lipid-modifying agents will continue to have a role in risk reduction, and due to recent clinical trials that have failed to demonstrate improved clinical outcomes with antihyperlipidemic therapies such as niacin, fibrates, and omega-3 fatty acids, the most appropriate second-line antihyperlipidemic drug therapies will remain unclear.[20-22] Therefore, the objective of this review is to summarize the clinical trial evidence evaluating the efficacy of second-line

antihyperlipidemic drug classes in reducing cardiovascular risk, provide recommendations for appropriate use of nonstatin lipid-altering drugs where clear evidence is lacking, and identify key areas of future research to support evidence-based lipid management.

Literature Search

English-language articles published through July 2012 were identified through the PubMed and Cochrane databases. Relevant ongoing trials were identified by searching www.ClinicalTrials.gov. Articles were identified by using the search terms statin, niacin, ezetimibe, fenofibrate, gemfibrozil, clofibrate, fibrate, cholestyramine, colestipol, colesevelam, bile acid sequestrant, omega-3 fatty acid, cholesterol ester transfer protein, proprotein convertase subtilisin/kexin 9, and clinical outcomes. Additional articles were identified through review of the reference sections of pertinent manuscripts. All English-language studies identified that evaluated the effect of niacin, ezetimibe, fibrates, bile acid sequestrants, or omega-3 fatty acids on clinical outcomes in patients at high risk for CVD events were assessed. Articles evaluating the efficacy of investigational or emerging therapeutics were also included.

Niacin

Niacin exerts its effects through inhibition of diacylglycerol acyltransferase-2, which decreases triglyceride synthesis, resulting in degradation of hepatic apolipoprotein B and lower circulating LDL cholesterol levels.[23] In addition, niacin reduces hepatic catabolism of high-density lipoprotein (HDL) cholesterol and is the most effective agent available for increasing HDL cholesterol levels.[23] Niacin results in a mean reduction in total cholesterol levels of 10%, LDL cholesterol levels of up to 15%, and triglycerides of 28% while increasing HDL cholesterol levels 23%.[24] The potent HDL cholesterol–raising effects of niacin make it a commonly prescribed agent for patients with suboptimal HDL cholesterol levels.

The first study to evaluate the effect of niacin on clinical outcomes was the Coronary Drug Project. Between 1966 and 1969 the Coronary Drug Project enrolled men with a history of myocardial infarction (MI) and randomized them to one of five lipid-lowering regimens or placebo. In the niacin arm of the study, immediate-release niacin 3000 mg daily reduced the incidence of nonfatal MI by 27% compared to placebo but showed no effect on the primary outcome of all-cause mortality.[25] However, an analysis of mortality data including fifteen years of patient follow-up was conducted nine years after trial discontinuation and showed a significant 11% reduction in all-cause mortality compared to placebo.[26]

The introduction of extended-release niacin products, which have fewer adverse effects, has renewed interest in niacin, and numerous clinical trials have evaluated extended-release niacin formulations. Multiple trials designed to assess surrogate endpoints have suggested that extended-release niacin may improve clinical outcomes in certain patient populations.[27-29] These findings are hypothesis generating and warrant follow-up with larger clinical trials; however, clinicians should be cautioned not to overinterpret the results from these small trials of short duration, which were not designed to evaluate clinical outcomes. The Atherothrombosis Intervention in Metabolic Syndrome with Low HDL Cholesterol/High Triglycerides: Impact on Global Health Outcomes (AIM-HIGH) trial failed to show an improvement with extended-release niacin compared to placebo.[20] The AIM-HIGH trial enrolled patients with established CAD and an atherogenic lipid profile (defined as HDL cholesterol levels <40 mg/dL for men or <50 mg/dL for women, triglyceride levels 150-400 mg/dL, and LDL cholesterol levels <180 mg/dL if not receiving a statin) and randomized them to simvastatin plus either niacin 1500-2000 mg daily or placebo (which contained 50 mg of niacin to help maintain blinding). After three years of follow-up, the trial was stopped early due to lack of efficacy and a higher number of ischemic stroke events in patients receiving niacin. No significant difference was observed in the primary endpoint (composite

of death from CHD, nonfatal MI, ischemic stroke, hospitalization for an acute coronary syndrome, or symptom-driven coronary or cerebral revascularization) which occurred in 282 patients (16%) receiving niacin and 274 patients (16%) receiving placebo.[20]

The Heart Protection Study 2–Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE [NCT00461630]) was designed to assess the effect of niacin-laropiprant (a prostaglandin D_2 receptor antagonist that reduces the incidence of flushing) on the primary outcome of nonfatal MI or coronary death, nonfatal or fatal stroke, or revascularization procedure. HPS2-THRIVE randomized over 25,000 patients to simvastatin 40 mg daily plus niacin-laropiprant or placebo. Preliminary results were released in December 2012, and indicate that the combination of niacin-laropiprant is not more effective than placebo.[30] These disappointing results are consistent with AIM-HIGH another study powered to evaluate clinical outcomes in patients receiving niacin while optimized on statin therapy.

In summary, clinical trials show that niacin effectively lowers LDL cholesterol and raises HDL cholesterol levels. Immediate-release niacin monotherapy has been shown to improve clinical outcomes, whereas studies demonstrating improved outcomes when niacin is used in combination with a statin are lacking, and although niacin is commonly prescribed to raise HDL cholesterol, the results of the AIM-HIGH trial demonstrate that this strategy does not improve clinical outcomes. Therefore, current data support the use of niacin to lower LDL cholesterol; however, as the use of niacin to raise HDL cholesterol levels has been evaluated and does not improve patient outcomes, it is not recommended.

Ezetimibe

Ezetimibe exerts its effects at the brush border of the small intestine through inhibition of Niemann–Pick C1-Like 1 (NPC1L1), a key mediator of cholesterol absorption.[31] Inhibition of NPC1L1 decreases cholesterol absorption and causes a compensatory increase in

cholesterol synthesis.[32] The net effect is a mean 19% reduction in LDL cholesterol and 4% increase in HDL cholesterol, with no significant changes in triglycerides.[33] Ezetimibe is commonly prescribed to lower LDL cholesterol levels in patients who have not achieved therapeutic goals despite optimization of statin therapy and to patients who are intolerant to statins. There are currently no clinical trials evaluating the effect of ezetimibe monotherapy on cardiovascular outcomes; however, the effect of ezetimibe in combination with simvastatin was investigated in the Study of Heart and Renal Protection (SHARP) trial, which compared ezetimibe 10 mg plus simvastatin 20 mg daily to placebo in patients with moderate-to-severe chronic kidney disease.[34] Patients receiving ezetimibe plus simvastatin had significantly lower incidence of the primary composite outcome of nonfatal MI or coronary death, nonhemorrhagic stroke, or any arterial revascularization procedure. However, the SHARP trial did not have a simvastatin monotherapy group, making it impossible to distinguish the effects of ezetimibe therapy from the effects of statin therapy. It should be noted that a higher incidence of cancer was observed in the simvastatin plus ezetimibe group in the Simvastatin and Ezetimibe in Aortic Stenosis (SEAS) trial, raising fears that ezetimibe use may be associated with increased cancer risk.[35] A meta-analysis published in 2008, which used data from SEAS, SHARP, and Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT), did not find that ezetimibe was associated with increased cancer risk.[36] At the time of publication, SHARP and IMPROVE-IT did not have complete follow-up; however, the final analysis of the SHARP did not report any association with cancer, suggesting that ezetimibe use is not associated with increased cancer risk.[34] The IMPROVE-IT trial is scheduled for completion in June 2013 and will compare simvastatin monotherapy to simvastatin plus ezetimibe in high-risk patients after an acute coronary syndrome.[37, 38] The primary outcome is the composite of cardiovascular death, nonfatal MI, hospitalization for unstable angina, revascularization, or stroke.. This direct comparison of simvastatin to a more intense lipid-lowering regimen of simvastatin plus

ezetimibe will provide much-needed evidence regarding the efficacy and safety of ezetimibe as an adjunct to statin therapy for reducing cardiovascular events in high-risk patients.

Collectively, the currently available evidence indicates ezetimibe is a safe and effective agent to lower LDL cholesterol as monotherapy or in combination with statin therapy; however, there are no clinical trials demonstrating that ezetimibe alone or as an adjunct therapy in combination with a statin improves clinical outcomes. Based on current data, the use of ezetimibe should be limited to patients who the clinician feels warrant additional LDL cholesterol lowering and who are either at their maximum-tolerated statin dose or are unable to adhere to statin therapy after multiple attempts.

Fibrates

Fibrates activate peroxisome proliferator-activated receptor α (PPARα),[39, 40] increasing transcription of multiple genes involved in cholesterol metabolism, including fatty acid–binding proteins.[41, 42] Transcription of fatty acid–binding proteins leads to an increase in lipolysis, induction of fatty acid uptake by the liver, reduction in triglyceride production, and an increase in HDL cholesterol production.[43] Fibrates produce a mean reduction of 15-20% in total cholesterol level, 32-45% in triglyceride level, and 3-20% in LDL cholesterol level, and a mean increase in HDL cholesterol of 6-16% in hyperlipidemic patients.[44, 45] As a result of their potent triglyceride-lowering effects, fibrates are frequently prescribed for patients with elevated triglyceride levels.

Clofibrate was developed in the 1960s whereas gemfibrozil and fenofibrate were developed more recently. Fibrates have been evaluated in multiple clinical trials, with inconsistent results. The World Health Organization study compared clofibrate to placebo in apparently healthy men with elevated cholesterol levels, and the clofibrate group had a lower incidence of major ischemic heart disease, which was driven by a lower incidence of nonfatal MI but had higher overall mortality rates.[46] These findings persisted in a follow-up

report that covered more than nine years of observation.[47] In addition, the clofibrate arm of the Coronary Drug Project trial did not show a significant difference in mortality or the combined endpoint of death due to coronary heart disease or occurrence of definite, nonfatal MI in patients receiving clofibrate compared to patients receiving placebo.[25] Similarly, no significant findings were observed in the fifteen-year follow-up study.[26]

The Helsinki Heart Study randomized 4081 apparently healthy men with non-HDL cholesterol ≥ 200 mg/dL to gemfibrozil 1200 mg daily or placebo. Use of gemfibrozil was associated with a significant 34% reduction in the incidence of CHD; however, no significant difference in mortality was observed.[48] In addition, the Veterans Affairs Cooperative Studies Program High-Density Lipoprotein Cholesterol Intervention Trial (VA-HIT) compared of gemfibrozil 1200 mg daily to placebo in men with a history of CHD; the primary composite outcome was incidence of nonfatal MI or death from CHD. Subjects randomized to placebo experienced 275 events (22%) whereas those randomized to gemfibrozil had 219 events (17%), representing a significant 22% relative risk reduction. Gemfibrozil use was also associated with nonsignificant reductions in death from CHD (22%, P=0.07) and overall mortality (11%, P=0.23).[49] The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study assessed the efficacy of fenofibrate in patients with type 2 diabetes (with or without existing coronary disease). In contrast to the Helsinki Heart Study and VA-HIT trials, randomization to micronized fenofibrate 200 mg daily did not significantly reduce the primary composite outcome of CHD death or nonfatal MI; however, a significant reduction in the secondary composite outcome of cardiovascular death, MI, stroke, and coronary and carotid revascularization was observed (11% relative risk reduction; P=0.04).[50] Similarly, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) lipid study compared fenofibrate 165 mg daily to placebo in patients with type 2 diabetes at high risk for CVD who were also being treated with simvastatin; no significant difference was observed in the primary

outcome of first occurrence of a major cardiovascular event.[21] A possible benefit in patients with high triglyceride levels and low HDL cholesterol levels was noted by the authors.

In summary, early trials evaluating fibrate monotherapy suggested that fibrates may improve clinical outcomes in men at moderate-to-high risk for CHD; however, more recent trials evaluating fibrates (with or without statin therapy) have not shown compelling evidence of benefit. Based on the current literature, we do not recommend the use of fibrates to lower LDL cholesterol, alone or in combination with a statin, unless other medications are not tolerated by the patient. The efficacy of fibrates in certain subsets of dyslipidemia, including individuals with high triglyceride and low HDL cholesterol levels, warrants clinical trial evaluation in order to define a clear role for fibrates in treatment of dyslipidemias.

Bile Acid Sequestrants

Bile acid sequestrants (BAS) include cholestyramine, colestipol, and colesevelam. These agents block bile acid reabsorption and reduce its recirculation to the liver, increasing conversion of cholesterol to bile acid through the LDL receptor pathway and lowering circulating LDL cholesterol levels.[51, 52] BAS result in a mean reduction in total cholesterol of up to 14% and LDL cholesterol of up to 21% while increasing HDL cholesterol 3% and triglycerides 2-5%.[53] Despite a positive effect on the overall lipid profile, BAS are not commonly prescribed secondary to multiple drug interactions and poor patient tolerance resulting in their frequent discontinuation.[54]

The Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT) was one of the first studies to convincingly demonstrate that reduction of circulating cholesterol levels with pharmacological agents could improve clinical outcomes in patients at high risk for CHD. Beginning in 1973, LRC-CPPT recruited 3806 men with elevated total and LDL cholesterol levels and no clinical manifestations of CHD. Individuals randomized to cholestyramine 24 g daily had a 19% reduction in the primary composite endpoint of definite

CHD death and/or definite nonfatal MI; however, all-cause mortality was not significantly different.[55] Similarly, in an early study of colestipol in 2278 patients with elevated total cholesterol levels, colestipol 5 g daily reduced CHD mortality compared to placebo in men but not in women.[56]

In summary, BAS have been shown to improve clinical outcomes when used as monotherapy, but have not been evaluated in combination with statin therapy. Currently, the use of BAS is limited due to their poor tolerability and frequent discontinuation by patients,[54] multiple drug interactions, and lack of trials demonstrating effectiveness when used in combination with statins. However, based on the currently available evidence, BAS are a reasonable choice to lower LDL cholesterol, either alone or in combination with a statin, in patients who are able to adhere to therapy. Additional trials are needed to determine if BAS improve clinical outcomes in patients receiving statin therapy in order to better define their appropriate role in cholesterol management.

Omega-3 Fatty Acids

Although omega-3 fatty acids (eicosapentaenoic acid–docosahexaenoic acid; EPA-DHA) do not lower LDL cholesterol, dietary supplementation with fish oils or use of prescription omega-3 acid ethyl esters is common in patients at high risk for cardiovascular events and in patients with elevated triglyceride levels. Although the mechanisms underlying the effects of omega-3 fatty acids on cholesterol profiles are not completely understood, they are known to reduce hepatic very low–density lipoprotein (VLDL) cholesterol and triglyceride synthesis and secretion as well as increase triglyceride clearance.[57] When taken in combination with statin therapy, prescription omega-3 acid ethyl esters produce a median decrease in total cholesterol of 5%, increases in LDL cholesterol of 1% and HDL cholesterol of 3%, and a 30% decrease in triglycerides.[58] Interestingly, there is evidence that purified EPA (icosapent

ethyl), which was recently approved for use in the United States, does not increase LDL cholesterol and actually reduces these levels.[59]

Studies evaluating omega-3 fatty acid supplementation include the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico (GISSI) trial, which enrolled post-MI patients between 1993 and 1995. The GISSI trial showed that supplementation of EPA-DHA resulted in a significant 10% reduction in risk for the combined primary endpoint of death, nonfatal MI, and nonfatal stroke ($P<0.05$); however, less than 5% of patients were taking statins at study entry.[60] The Japan EPA Lipid Intervention Study (JELIS) randomized Japanese patients with elevated cholesterol levels to statin therapy alone (pravastatin 10 mg/day or simvastatin 5 mg/day) or EPA plus a statin. The combined endpoint of major coronary events was reduced 19% in the EPA group ($P=0.01$), demonstrating that EPA supplementation can reduce cardiovascular morbidity when used in combination with statin therapy.[61] However, it should be noted that the statin doses were much lower than those currently used in clinical practice in the United States, and the study exclusively enrolled Japanese patients, who have high dietary consumption of omega-3 fatty acids. The Alpha Omega Trial randomized patients who had experienced an MI within the previous 10 years to supplementation with omega-3 fatty acids or placebo and showed no difference in the primary endpoint of major cardiovascular events.[62] Similarly, the OMEGA trial randomized patients with a recent MI who were receiving current standard of care treatments to omega-3 acid ethyl esters or placebo and showed no difference in the primary endpoint of rate of sudden cardiac death.[22] Omega-3 fatty acid supplementation was also evaluated in a population at high risk for cardiovascular events who had impaired fasting glucose, impaired glucose tolerance, or diabetes mellitus in the Outcome Reduction with an Initial Glargine Intervention (ORIGIN) trial. No significant reduction was noted in the primary outcome of death from cardiovascular causes (9.1% vs. 9.3%) or the secondary endpoint of major vascular events (16.5% vs. 16.3%) in patients randomized to omega-3 fatty acids compared

to patients randomized to placebo.[63] Of note, statin use was highly prevalent in both the Alpha Omega and OMEGA trials, with greater than 80% of subjects receiving statins, and to a lesser extent the ORIGIN trial, with over 50% of patients receiving statins. The negative results of these trials raise concerns regarding the efficacy of omega-3 acid ethyl esters when used in patients who are optimized on statin therapy. In addition, a recent meta-analysis of trials evaluating the effect of omega-3 fatty acid supplementation on risk of major CVD events did not show a significant reduction in all-cause mortality, cardiac death, or any cardiovascular events.[64] The Reduction of Cardiovascular Events Outcomes Trial (REDUCE-IT) will assess the effectiveness of purified EPA to improve cardiovascular outcomes in high-risk patients with elevated triglycerides who are optimized on statin therapy. Collectively, clinical trials have demonstrated that omega-3 acid ethyl esters are safe and may reduce the risk of cardiovascular events in certain populations. Additional clinical trials are necessary to evaluate potential disparities in the effects of purified EPA and EPA-DHA on cardiovascular outcomes.

Roles of Antihyperlipidemic Drug Classes Beyond the First-Line Statins in Reducing Cardiovascular Risk

Cardiovascular disease remains a major public health problem in the United States and other developed countries, and reduction of LDL cholesterol improves prognosis in patients at high risk for cardiovascular events. Based on current evidence, statins will remain the first-line therapy of choice for the foreseeable future; however, the roles of antihyperlipidemic drug classes beyond statins remains unclear. New treatment guidelines will continue to place an emphasis on evidence-based data to support recommendations, making the optimal second-line therapeutic regimen controversial due to recent clinical trials assessing antihyperlipidemic therapies that have not demonstrated a benefit. Given the

significant number of patients unable to adhere to statin therapy due to the occurrence of drug-related adverse events, particularly at high doses,[65] and the persistence of high LDL cholesterol levels in some patients receiving high-dose statins, second-line antihyperlipidemic medications will continue to represent an unmet medical need in the management of patients at high risk for cardiovascular events.

Given the evidence base supporting their use in reducing cardiovascular events, it is important that patients be given an adequate trial of statin therapy and titrated to maximum-tolerated doses prior to adding adjunctive therapies to achieve desired LDL cholesterol levels. In addition, all patients should be counseled on proper diet and exercise regimens to reduce cardiovascular risk. When initiating statin therapy, we recommend that you thoroughly counsel patients on the known risks and benefits of treatment, and provide focused education to build long-term adherence to therapy. It is important to review past experience with specific agents and acknowledge issues of formulary access and patient preferences in order initiate the statin regimen that patients are most likely to accept. In cases of past experience of statin intolerance, it may be necessary to employ alternate-day dosing or thrice-weekly dosing and gradually increase the frequency and dose as needed to optimize treatment benefits.[66]

In high-risk patients who require LDL cholesterol–lowering therapy but are unable to tolerate statin therapy in spite of adequate trials with more than one agent, we generally recommend ezetimibe or niacin as an LDL cholesterol–lowering approach. Although ezetimibe is lacking clinical outcomes data in support for its use, it is well tolerated and effectively lowers LDL cholesterol. Niacin and BAS also effectively lower LDL cholesterol and each has been shown to improve clinical outcomes in CAD patients, making them reasonable evidence-based choices that should be considered in all patients who require a second-line antihyperlipidemic therapy. [25, 26, 55] However, it should be noted that the Coronary Drug Project used an immediate-release formulation of niacin, and both the

Coronary Drug Project and LRC-CPPT were conducted when the standard of care in cardiology was vastly different than it is today. Given the issues of potential intolerance with niacin and BAS,[54] ezetimibe is a practical choice in select patients in whom the clinician feels that LDL cholesterol lowering is warranted and other medications are not tolerated.

A significant number of patients will either fail to achieve adequate LDL cholesterol lowering or experience cardiovascular events in spite of adhering to high-dose statin therapy. Although several antihyperlipidemic drugs have been evaluated in combination with statin therapy, to date, none have demonstrated improved outcomes when compared to placebo (Table 1).[20, 21, 34, 38] As with patients who are unable to tolerate statin therapy, ezetimibe and niacin are effective agents to lower LDL cholesterol levels in patients already receiving a statin. The IMPROVE-IT trial will provide much-needed data evaluating the efficacy of ezetimibe to improve clinical outcomes when used in combination with statin therapy. Niacin effectively lowers LDL cholesterol in patients treated with statins and may improve outcomes in this patient population; however, clinical trials evaluating niacin in combination with statin therapy have been limited to patients who have achieved adequate LDL cholesterol lowering but have low HDL cholesterol levels. Since the currently available evidence from these trials has demonstrated that niacin does not improve clinical outcomes when prescribed to raise HDL cholesterol, this practice is not evidence based and therefore is not recommended. Similar to ezetimibe and niacin, BAS effectively lower LDL cholesterol but have not been shown to improve outcomes when used in combination with statins; they remain a reasonable choice in patients who are able to maintain adherence when the clinician feels that combination therapy to lower LDL cholesterol is warranted. Fibrates have been evaluated in patients receiving statin therapy and have failed to improve clinical outcomes; therefore, the use of fibrates is not recommended unless patients are unable to tolerate alternative agents. Although omega-3 fatty acids do not lower LDL cholesterol levels, they have been shown to improve clinical outcomes in CAD patients in some studies;

however, clinical trials evaluating omega-3 fatty acids in combination with statins have not demonstrated benefit, and therefore their routine use is not recommended in the absence of elevated triglyceride levels. It should be noted that purified EPA has been shown to decrease LDL cholesterol levels, and the differing effects of purified EPA and EPA-DHA may impact clinical outcomes. The ongoing REDUCE-IT trial will provide data evaluating the effects of purified EPA on cardiovascular outcomes.

The substantial residual risk of cardiovascular events in patients receiving standard of care therapy highlights the need for improved lipid management strategies to reduce cardiovascular risk. Several novel therapeutic strategies are currently under investigation such as cholesterol ester transfer protein (CETP) and proprotein convertase subtilisin–kexin 9 (PCSK9) inhibition. To date, two CETP inhibitors have failed phase III clinical trials.[67, 68] The ongoing Randomized Evaluation of the Effects of Anacetrapib Through Lipid-Modification (REVEAL) trial (Table 1) will provide additional insight into the clinical utility of CETP inhibitors (NCT01252953). The development of PCSK9 inhibitors is still in its infancy; however, results from phase I trials are encouraging.[69] These investigational agents require rigorous evaluation in clinical trials to evaluate their safety and efficacy prior to FDA approval and use in lipid management. In addition to the investigation of new drug targets and therapies, current and future research efforts should focus on improving the precision in which we assess risk. This includes clarification of the role of traditional biomarkers of cardiovascular risk including LDL cholesterol, HDL cholesterol, and triglyceride levels, and investigation of additional biomarkers such as apolipoprotein B, LDL cholesterol particle size, and biomarkers of inflammation. In addition, a personalized medicine approach using genetic or physiologic biomarker data may prove more efficacious in the management of CVD than current lipid management strategies, which are based on global risk.

In summary, the currently available evidence clearly supports the use of statins as the first-line lipid management strategy to lower LDL cholesterol levels and reduce

cardiovascular risk, whereas alternative antihyperlipidemic agents have less evidence supporting their ability to improve clinical outcomes. It should be noted that LDL cholesterol levels are a surrogate marker for cardiovascular risk, and the degree of LDL lowering may be an indirect marker of drug efficacy. However, use of second-line agents to achieve adequate LDL cholesterol lowering is reasonable in patients who are unable to tolerate statins or who do not achieve desired cholesterol levels while taking maximum-tolerated doses of statins.[15] Current and future research efforts will focus on novel therapeutics and risk-assessment strategies, including alternative biomarkers of cardiovascular risk and drug efficacy. Findings from these studies may ultimately improve the available selection of pharmacologic agents and challenge our current understanding of lipid management.

Conclusion

We have summarized the current evidence supporting the use of second-line antihyperlipidemic therapies, provided clinical recommendations for the appropriate use of these therapies, and identified important areas for future research in order to improve lipid-based management of patients and reduce the burden of CVD. Given the complexity, magnitude, and burden of CVD, opportunities to improve processes of care and identify new therapeutic options clearly exist.

Appendix II References

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Table 1. Summary of Clinical Trials Evaluating Combination Therapy with Statins

Trial	Population	Intervention	Primary Outcome	Results
SHARP[34]	Patients with chronic kidney disease	Simvastatin + ezetimibe vs. placebo	Nonfatal MI or coronary death, nonhemorrhagic stroke, or revascularization	Simvastatin + ezetimibe reduced primary outcome
ACCORD[21]	Patients with type 2 diabetes mellitus	Simvastatin + fenofibrate vs simvastatin + placebo	First occurrence of nonfatal MI, nonfatal stroke, or death from cardiovascular causes	No significant difference in the primary outcome
AIM-HIGH[20]	Patients with CAD and an atherogenic profile (secondary CAD prevention)	Simvastatin + niacin vs simvastatin + placebo	Death from CHD, nonfatal MI, ischemic stroke, hospitalization for ACS, or symptom-driven revascularization	Stopped early due to lack of efficacy
IMPROVE-IT[38]	Stabilized, high-risk patients after an ACS	Simvastatin + ezetimibe vs simvastatin + placebo revascularization, or stroke	Cardiovascular death, nonfatal MI, hospitalization for UA, 2013	Study completion anticipated in June
HPS2-THRIVE	Patients with CAD, PAD, diabetes mellitus, or CHD	Niacin-laropiprant vs. placebo in patients receiving simvastatin	Nonfatal MI or coronary death, nonfatal or fatal stroke, or revascularization	Preliminary results indicate no difference in the primary endpoint
REDUCE-IT	Patients with elevated triglyceride levels and CHD or at least one risk factor for CHD	Icosapent ethyl (EPA ethyl ester) vs. placebo in patients receiving statins	Cardiovascular death, MI, stroke, coronary revascularization, or hospitalization for UA	Study completion anticipated in 2016
REVEAL	Patients ≥ 50 years old with symptomatic CHD and history of MI, cerebrovascular disease, PAD, or diabetes mellitus	Anacetrapib vs. placebo in patients receiving atorvastatin	Cardiovascular death, MI, or coronary revascularization	Study completion anticipated in 2017

SHARP = Study of Heart and Renal Protection; ACCORD = Action to Control Cardiovascular Risk in Diabetes; AIM-HIGH = Atherothrombosis Intervention in Metabolic Syndrome with Low HDL Cholesterol/High Triglycerides: Impact on Global Health Outcomes; IMPROVE-IT = Improved Reduction of Outcomes: Vytorin Efficacy International Trial; HPS2-THRIVE = Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events; REDUCE-IT = Reduction of Cardiovascular Events Outcomes Trial; REVEAL = Randomized Evaluation of the Effects of Anacetrapib Through Lipid-Modification; MI = myocardial infarction

APPENDIX III

PERSONAL CONTRIBUTIONS TO DISSERTATION CHAPTERS

Analyses presented in Chapters II and III utilized the Supporting a Multidisciplinary Approach to Researching Atherosclerosis (SAMARA) cohort. Research subjects were enrolled in the SAMARA cohort and RNA expression data was generated by members of Dr. Cam Patterson's laboratory. Utilizing this dataset, I subsequently contributed to this project by developing the hypotheses tested in Chapters II and III, generating the plasma inflammatory biomarker data in collaboration with the UNC Cytokine Analysis Facility, conducting the statistical analysis for the candidate biomarker comparison, conducting the functional annotation analysis, analyzing and interpreting the data, and writing each chapter.

Research subjects were enrolled in the coronary artery disease cohort utilized in Chapter IV, by multiple members of Dr. Craig Lee's laboratory. My contributions to this project include enrolling a portion of the patients and assisting in study visits, developing the hypotheses tested in the analysis, quantifying plasma eicosanoid levels, working with the UNC Cytokine Analysis Facility to quantify circulating biomarkers of inflammation, designing and conducting the statistical analyses, analyzing and interpreting the data, and writing the chapter.

The preclinical studies included in Chapter V were designed and conducted by myself in collaboration with Dr. Craig Lee. My contributions to this project include developing the hypotheses tested, generating the phenotypic data presented in the chapter, analyzing and interpreting the data, and writing the chapter.

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